Volume 21 Sept. 1957 No. 5

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

CONTENTS

Published by the Agricultural Chemical Society of Japan

c/o Faculty of Agriculture, University of Tokyo

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $ 6.00 for all oversea subscribers including postage fee. Please address orders to the sole agent.

The Agricultural Chemical Society of Japan also published the Journal of the Agricultural Chemical Society of Japan (Nippon Nogei Kagakukaishi), printed in Japanese.

The Agricultural Chemical Society of Japan

Sole Agent
Maruzen Co., Ltd.
P. O. Box 605, Tokyo Central
Tokyo, Japan,

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 267~273, 1957]

Phenolsulphatase Synthesis with Tyramine as a Inducer by *Aerobacter aerogenes*

By Tokuya HARADA

Institute of Scientific and Industrial Research, Osaka University

Received March 4, 1957

The phenolsulphatase of *terobacter aerogenes* was found to be formed in cell suspensions containing tyramine and casein hydrolyzate under aerobic conditions in a few hours.

Factors affecting phenolsulphatase synthesis in cell suspensions were studied.

The relationship between the rate of enzyme activity formed and the level of tyramine concentration in 10^{-4} M to 10^{-3} M is linear. The optimum concentration of tyramine for its utilization and phenolsulphatase induction is in both cases 5×10^{-3} M. The opt. concentration of casamino acids for phenolsulphatase induction is 0.2 per cent and the opt. pH is 7.2. Agents such as 2,4-dinitrophenol, sodium azide, arsenate and potassium cyanide inhibit the induction of this enzyme. The external inducer of phenolsulphatase should be highly specific. On addition of tyramine, hydroxytyramine and noradrenalin are effective, although the effect of noradrenalin is very weak.

It was found by us that *Aerobacter aerogenes*, strain 9621, failed to produce phenolsulphatase (hereafter, referred to as PSase) on a synthetic medium unless the medium was supplemented with an unidentified principle contained in some commercial peptone preparations, yeast extract or hen's liver powder[1]. Thereafter, this principle in these preparations was decided as to be tyramine[2,3]. PSase was produced by strain 9621 only when tyramine or hordenine was added to the medium. It was further shown that in the case of the other two strains, F 50 and 8329 ATCC, although slight PSase activity developed in the absence of tyramine, tyramine stimulated the production of PSase markedly, while hordenine showed no influence.

As will be reported in this paper, a preliminary study indicated that strain 9621 produced PSase employing tyramine as the sole source of carbon and nitrogen. However, it would be desirable to use a cell suspension, in order to study further factors affecting PSase production and the biochemical mechanism involved. The use of a cell suspension would clarify whether tyramine is concerned with the net PSase synthesis or not.

It was soon learned that shaking the flasks containing cell suspension, tyramine and casamino acids, in an atmosphere for a few hours, produced the PSase significantly. Further studies indicated that tyramine induced the net PSase synthesis. These results and various factors affecting PSase synthesis under these conditions will be demonstrated in the present paper.

METHODS

Estimation of PSase Activity of Various Cultures. Ten ml of the basal medium contained the following reagents: 10 mg of tyramine hydrochloride, 5 mg of KH_2PO_4, 5 mg of K_2HPO_4, 2 mg of $MgSO_4 \cdot 7H_2O$, 0.1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $MnSO_4 \cdot 4H_2O$ and 0.1 mg of NaCl.

In this basal medium, tyramine is the sole source of carbon and nitrogen for growth. A quantity of 50 mg of each of the following i.e., $CaCO_3$, glucose, sodium citrate and Bacto casamino acids was added solely or

1) T. Harada and K. Kono, *J. Agr. Chem. Soc. Japan*, **29**, 57 (1955).

2) T. Harada, *J. Agr. Chem. Soc. Japan*, **29**, 874 (1955).

3) T. Harada and F. Hattori, This Bulletin, **20**, 110 (1956).

in various combinations as shown in Table I. The culture for the inoculum was prepared by transferring one loop from the agar-stab to 10 ml of broth. A twenty four hour-culture in this medium was used dropwise to inoculate each tube. After 6 days' incubation at 34°C, one ml of each culture medium was neutralized with 0.1 M HCl or NaOH and was filled up to 2 ml with distilled water. The method for the determination of PSase was the same as previously reported[4].

Estimation of PSase Induction by Cell Suspensions. The medium which was used to obtain cells for the PSase synthesis, had the following composition: Bacto casamino acids, 0.5 per cent; KH_2PO_4, K_2HPO_4, 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl, 0.001 per cent; pH 7.2. Resting cell suspensions were obtained by growing *Aerobacter aerogenes*, strain 9621 ATCC, in this medium at 34°C for 48 hours, using a one per cent inoculum from a yeast extract broth culture. It should be noted that PSase producing capacity decreased if the obtaining of the cells was prolonged for more than three days. Harvesting was accomplished by centrifugation, the cells washed thrice with distilled water and the final cell suspension was adjusted to a concentration of a 5 mg dry cell material per one ml with 0.2 M phosphate buffer, pH 7.2.

To test the PSase induction, 4 ml of each test solution was added into 4 ml of this cell suspension and the mixture aerated on a reciprocal shaker at 34°C. A quantity of 0.5 ml of the mixture was pipetted at appropriate intervals and the cells centrifuged. The supernatant showed no PSase activity. The cells, thus obtained were suspended into one ml of water and this suspension was transferred to a graduated test tube which had already contained 2 ml of acetate buffer, pH 7.2 and one-ml of 0.005 M *p*-nitrophenyl sulphate solution and had been preliminary incubated at 34°C for five minutes. The content of the tube was immediately brought to a final volume of 5 ml with distilled water and was incubated further under occational shaking for 20 minutes at 34°C. The amount of *p*-nitrophenol produced by the enzymatic action was estimated according to the method already mentioned[4]. PSase activity is expressed as μg of *p*-nitrophenol produced per one hour at 34°C by cells in one-ml of the reaction mixture inducing enzyme. Turbidity of the reaction mixture was determined by a Kotaki AKA photoelectric colorimeter using a S-66 filter. One-ml of the reaction mixture which showed turbidity equivalent to the optical dencity of 0.35, contained approximately 0.5 mg of the dried cell.

Chemicals. Tyramine hydrochloride was purchased from L. Light and Co., Ltd.; hordenine hydrosulphate and adrenalin from Merk and Co.; noradrenalin, phenylethylamine and tryptamine hydrochloride from Tokyo Kasei Co.; ephedrine from Tachiyama Kasei Co.; serotonin from California Foundation for Biochem. Research; *o,m,p*-aminophenol from Wako Pure Chem. Ind. Ltd. The author is indebted to Dr. T. Kametani for the preparation of hydroxytyramine and to Dr. M. Isono for the preparations of *p*-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid; also for the sympatol which was kindly supplied by Dr. H. Matsumoto. Bacto casamino acid was used as a casein hydrolyzate. *p*-Nitrophenyl sulphate as an enzyme substrate, was synthesized according to the modified methods of Whitehead et al.

RESULTS AND DISCUSSION

PSase Production in a Culture Medium Containing Tyramine as the sole Source of Carbon and Nitrogen. Our previous report showed that *A. aerogenes* 9621 produced PSase when grown on a medium composed of 0.5 per cent casein hydrolyzate, inorganic salts and tyramine or hordenine. An attempt was made to learn whether this organism does produce PSase in a culture medium containing tyramine as the sole source of carbon and nitrogen comparing with other media composed of both tyramine and other carbon or nitrogen sources. As shown in Table I, *A aerogenes*

TABLE I
PHENOLSULPHATASE ACTIVITY OF *AEROBACTER AEROGENES* CULTURED IN VARIOUS MEDIA CONTAINING TYRAMINE.

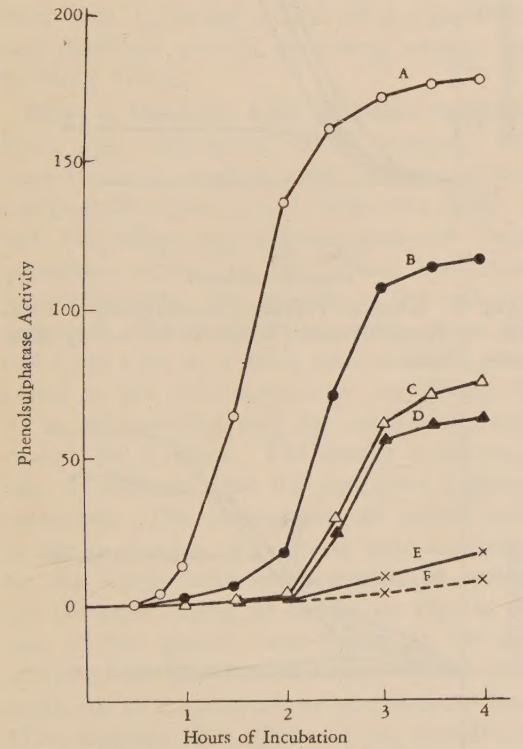
Medium	Final pH	Phenolsulphatase Activity
Basal (10 ml)*	6.2	400
plus 50 mg $CaCO_3$	6.6	465
plus 50 mg glucose	4.2	0
plus 50 mg glucose, 50 mg $CaCO_3$	6.2	180
plus 50 mg sodium citrate	7.8	420
plus 50 mg Bacto casamino acids	7.8	360

Incubation time, 6 days. Basal medium contained 10mg of tyramine HCl, 5mg of KH_2PO_4, 5mg of K_2HPO_4, 2mg of $MgSO_4 \cdot 7H_2O$, 0.1mg of $FeSO_4 \cdot 7H_2O$ 0.1mg of $MnSO_4 \cdot 4H_2O$ and 0.1mg of NaCl.

4) T. Harada and K. Kono, *J. Agr. Chem. Soc. Japan*, **28**, 608 (1954).

9621 grew well and produced PSase during 6 days in the medium containing tyramine as a sole source of carbon and nitrogen as well as in other media containing $CaCO_3$, sodium citrate or Bacto casamino acids besides tyramine. However, growth in the medium containing tyramine only was less than that of other media. In case of glucose with tyramine PSase producing capacity decreased to zero, but $CaCO_3$ restored this decrease to some extent.

Induction of PSase Synthesis with Tyramine. The cell suspension prepared as described in the experimental methods was devoid of PSase activity owing to the absence of tyramine in the culture medium. Attempts to induce PSase synthesis in them were made,



A 0.1% Casamino acids, 10^{-3}M Tyramine
B 0.1% Casamino acids, 0.1% Glucose, 10^{-3}M Tyramine
C 0.1% Glucose, 0.03% NH_4Cl, 10^{-3}M Tyramine
D 0.1% Glucose, 10^{-3}M Tyramine
E 0.03% NH_4Cl, 10^{-3}M Tyramine
F 10^{-3}M Tyramine

FIG. 1. Formation of Phenolsulphatase of *Aerobacter aerogenes* 9621 with Tyramine and other Reagents.

shaking these cell suspensions with tyramine and other reagents. The results of a typical experiment are presented in Fig. 1. When the cell suspension was aerated with tyramine and Bacto casamino acids on a reciprocal shaker, the development of activity began within 45 minutes and gradually accelerated to reach a steady vigorous rate within a few hours. In anaerobic conditions, no appreciable activity appeared even after 24 hours. With tyramine alone or tyramine and ammonium chloride, PSase activity were very weak. Accordingly, ammonia could not replace casein hydrolyzate, at least during period of a few hours. On the other hand, the cells, cultured 6 days on the medium composed of tyramine and inorganic salts, showed a high PSase activity as above mentioned. A definite answer for this reason cannot yet be given. It is known, however, that *A. aerogenes* produce and excrete amino acids during its growth in media of ammonium salts. Therefore, *A. aerogenes* may slowly produce an excess of amino acids, both in quantity and quality, over and above its growth requirements from tyramine. On accumulation of enough amino acids, the cells might produce PSase if tyramine is present.

Addition of glucose decreased PSase activity in the presence of Bacto casamino acids with tyramine. Since the final-pH of the reaction mixture did not decrease detectably, the effect of glucose may not only be due to the decrease of pH of the reaction mixture as observed in the case of prolonged cultivation of Table I. Moreover, addition of glucose to tyramine increases PSase activity when Bacto casamino acids are absent. When glucose is given with tyramine only or with ammonium salts, amino acids would be produced easier than in the case of cell suspensions without glucose, Therefore, PSase activity would increase to some extent. Thus, it appears that *A. aerogenes* needs both tyramine and proper amino acids at the same

time for the synthesis of PSase. The same behaviour of the nitrogen requirement was reported in the hydrogenlyase synthesis of *E. coli*,[5] in maltozymase synthesis of yeast[6] and in other cases.

Effect of Tyramine on PSase Synthesis. Cell suspensions were prepared with 0.2M phosphate buffer solution. To these suspensions 0.1 per cent Bacto casamino acids and varying concentrations of tyramine from 5×10^{-5} to 1×10^{-3} M, as final concentrations, were added. The mixtures were then aerated for 4 hours, after which the cells were centrifuged and their PSase activities were determined. As shown in Fig. 2, the enzyme activity increased linear against the concentration of tyramine added. Within 4 hours, the maximum PSase activity was obtained in each vessel and this activity retained several hours further.

In order to find out the optimum concentration of tyramine for the PSase induction, attempts were made to test the effect of the varying concentration of tyramine on the PSase induction by varying the shaking times.

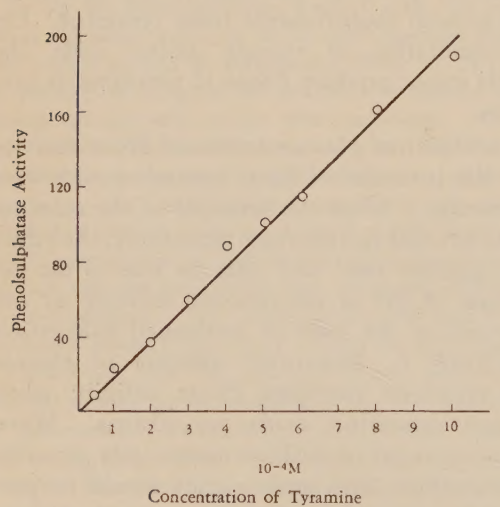


FIG. 2. Effect of Varying Concentrations of Tyramine on Phenolsulphatase Synthesis.

Simultaneously, the effect of tyramine on growth was observed by the estimation of the optical density of the turbidity of a 5-times diluted reaction mixture at appropriate intervals. Bacto casamino acids were added to 0.1 per cent, as the final concentration. The results are shown in Fig. 3 and Fig. 4. Tyramine at a concentration level of 5×10^{-3}M induced the PSase and stimulated growth the most in the earlier incubation-period, although

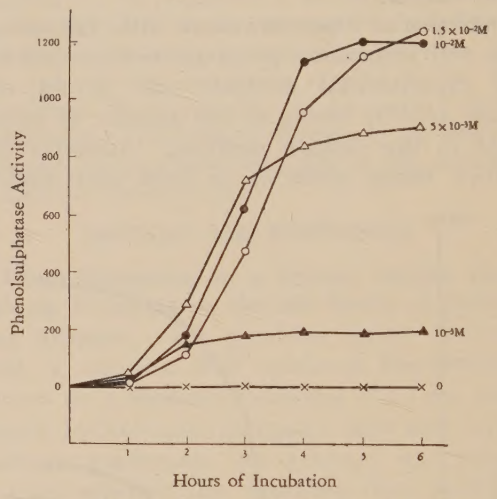


FIG. 3. Effect of Varying Concentrations of Tyramine on Phenolsulphatase Synthesis at Varying Incubation Times.

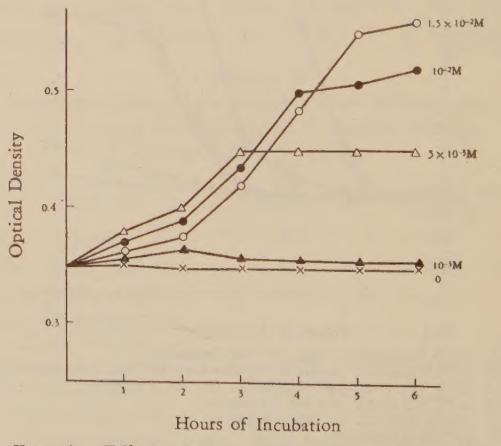


FIG. 4. Effect of Varying Concentrations of Tyramine on Growth at Varying Incubation Times.

5) M.J. Pinsky and J.L. Stokes, *J. Bact.*, **64**, 151 (1952).
6) H. O. Halvorson and S. Spiegelman, *J. Bact.*, **65**, 601 (1953).

the maximum PSase activity and growth were proportional to a concentration up to 1.5×10^{-2} M. This indicates that the optimal concentration of tyramine for both the PSase induction and the growth is about 5×10^{-3} M. The maximum PSase activity at a concentration of 5×10^{-3} M was about five-fold higher than at the concentration of 10^{-3} M. Even at such a concentration, the fact that the maximum PSase synthesis is linear between the activity and the concentration, was observed. At higher concentration the linear relationship could not be observed. PSase synthesis began after slight growth and appeared to continue as long as the bacteria grew in the presence of tyramine. Both PSase induction and growth ceased to increase when all the tyramine was consumed. It indicates, therefore, that tyramine would induce the PSase and stimulate growth, providing energy and building blocks.

Effect of Casamino Acids on PSase Synthesis. Since cell suspensions of *A. aerogenes* 9621 were found to produce little enzyme without casein hydrolyzate, an attempt was made to test the effect of concentration of Bacto casamino acids on the PSase induction. Simultaneously, the growth of the cells was observed. Bacto casamino acids, from 0.02 to 0.4 per cent as a final concentration, were added to the cell-suspensions containing 5×10^{-3} M of tyramine and the suspensions were shaken for 5 hours. The results as shown in Fig. 5, indicate that 0.2 per cent is almost optimum. The importance of amino acids in the formation of enzymes was supported by the works with other enzymatic system. On the other hand, as shown in Fig. 6, the rate of the growth was found to be proportional to the concentration of casamino acids, up to the concentration examined here. This suggests that there is no direct correlation between the rate of growth and the degree of PSase synthesis. Accordingly, it may be of interest to establish which of the component amino acids are responsible for

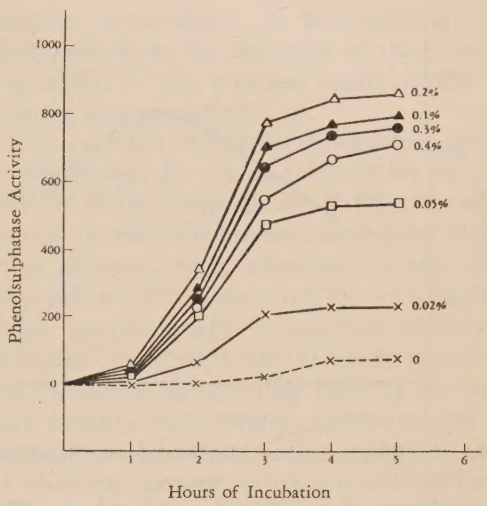


FIG. 5. Effect of Varying Concentrations of Casamino Acids on Phenolsulphatase Synthesis at Varying Incubation Times.

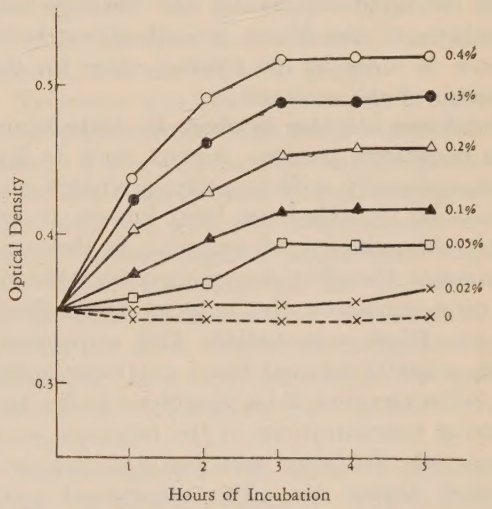


FIG. 6. Effect of Varying Concentrations of Casamino Acids on Growth at Varying Incubation Times.

PSase synthesis.

Effect of pH on PSase Synthesis. Each two-ml of cell suspension was mixed with one-ml of 0.4 M phosphate buffer at various pH values and one-ml of 0.4 per cent Bacto casamino acids solution containing 2×10^{-2} M tyramine. The mixture were shaken for 2 hours and

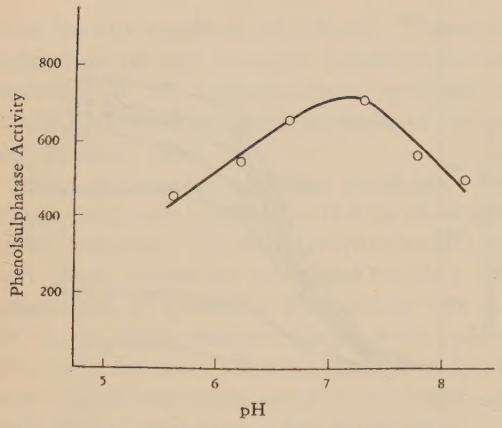


FIG. 7. Effect of pH on Phenolsulphatase Synthesis.

the cells were centrifuged and washed. The PSase assays were carried out on aliquots of each cells. The results obtained in this experiment are shown in Fig. 7. It is evident that the optimum pH for the induced biosynthesis of the PSase is approximately 7, which is close to the optimal value for the activity of the enzyme[4].

Inhibition of PSase Synthesis by Assimilatory and Respiratory Poisons. Agents such as 2,4-dinitrophenol[7], sodium azide, arsenate[8] and potassium cyanide have been known to inhibit the induction of enzyme. In these experiments, the effect of varying concentrations of these agents on the induced biosynthesis of the PSase was studied. Cell suspensions containing 0.1 per cent Bacto casamino acids, 5×10^{-3} M tyramine, 0.1 M phosphate buffer and varying concentrations of the inhibitor were employed. Inhibitor solutions had been neutralized before use. The suspensions were shaken for a period of 4 hours and afterwards the cells were centrifuged and washed twice with distilled water and assayed for PSase activity. The results obtained are shown in Table II. It is demonstrated that all these

TABLE II
INHIBITION OF PHENOLSULPHATASE SYNTHESIS

Additions (M)	Inhibition %
Sodium azide	
0.00025	26
0.0025	100
Sodium arsenate	
0.06	16
0.25	100
2,4-Dinitrophenol	
0.00025	54
0.0006	100
Potassium cyanide	
0.00025	30
0.0025	100

compounds inhibited the synthesis of PSase, and this inhibition was a function of the concentration of the inhibitor employed. Thus, PSase induction was found to be sensitive to assimilatory or respiratory poisons.

High Specificity of Tyramine as an External Inducer. Since from the results of the experiments mentioned above PSase was found to be induced by tyramine, being utilized as an energy source and building blocks, it might be possible that a metabolic substance derived from tyramine is concerned with the synthesis of the enzyme. Therefore, the effect of *p*-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid, on the PSase induction, which are assumed to be an oxidation intermediate of tyramine[10], was tested. Both substances were found to be ineffective. Thus, it would appear that the external inducer must possess a tyramine related constitution. A number of compounds related to tyramine which have been known hitherto, were tested as inducers of PSase. As shown in Table III, in addition to tyramine, hydroxytyramine and noradrenalin were effective although the activity of the latter was very weak. It is very striking that inductive activity is associated with the presence of constitution of tyramine. The noninducers include a variety of compounds; hordenine, sympatol, adrenalin,

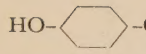
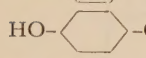
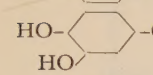
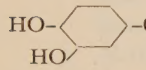
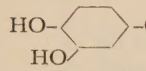
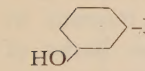
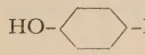
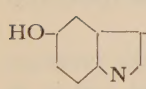
7) J. Monod, *Ann. Inst. Pasteur*, **70**, 384 (1944).

8) M. Sussman and S. Spiegelman, *Arch. Biochem.*, **29**, 54 (1950).

9) J. Fukumoto and T. Yamamoto, *Symposia on Enzyme Chemistry Japan*, **7**, 36 (1952)

10) J.B. Sumner and K. Myrback, *The Enzyme*, Vol. II, p. 536 (1952).

TABLE III
INDUCING ACTIVITY OF COMPOUNDS RELATED TO TYRAMINE.

Compounds	Inducing Activity
	190
	0
	0
	165
	6
	0
	0
	0
	0
	0
	0
	0
	0

Maximal phenosulphatase activity of organisms incubated in presence of compound (10^{-3}M).

phenylethylamine, ephedrine, tryptamine, *o*, *m*,*p*-aminophenol and serotonine. Though hordenine was ineffective in this experiment it was effective during the long incubation-period in the culture medium as previously reported[3]. This fact suggests that hordenine could not be converted into tyramine in cell suspensions under these conditions. It is not clear however, whether tyramine added to the reaction mixture might be converted to hydroxytyramine or not. Without such an inducer, urineindican or *p*-nitrophenyl sulphate, which are the substrate of the enzyme, was added to the reaction mixture, but no enzyme was produced.

The external inducer of this enzyme should be highly specific. Such a specificity may be seen in PSase synthesis of all PSase positive strains in *Enterobacteriaceae*, since some strains of *A. aerogenes*, *Salm. schottmuelleri* and *Salm. paratyphi* were demonstrated by us to produce PSase proportionally in response to varying preparations of peptone. It has been pointed out by Monod et al.[11] that there is no necessary identity between the substrate and inducer; some substrates are wholly incapable of eliciting enzyme synthesis, and inducers are known that cannot act as substrate but are structually related to it. Tyramine is, of course, not the substrate of the PSase, but it is similar with the structure of the substrate in respect of the presence of phenol. The inducer which is not substrate, but highly specific, has not yet been known.

Tyramine and hydroxytyramine are known to be pressor substances similar in action to, though less potent than adrenalin. Tyramine is formed from tyrosine by intestinal bacteria and absorbed into the blood stream of the host. Hydroxytyramine arises from "dopa" by "dopa" decarboxylase and is converted to adrenalin in mammals. Noradrenalin is present in some tissues of mammals with adrenalin. It is very interesting that such a compound is concerned with the synthesis of the enzyme.

Acknowledgement The author wishes to express his sincere thanks to Prof. Z. Nikuni for his kind advice and suggestions rendered throughout the course of this study and to Prof. K. Ichihara, Prof. S. Akabori, Prof. G. Terui and Dr. S. Naono for their valuable advices.

11) J. Monod, G. Cohen-Bazire and M. Cohn, *Biochem. et Biophys. Acta*, **7**, 585 (1951).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 274~278, 1957]

The Chemistry of Ilexol. I.

Oxidation of Ilexol. Formation of Ilexone

By Kazuyoshi YAGISHITA and Syun ISEDA

*Department of Chemistry, Kumamoto Women's University**

Received March 16, 1957

Ilexol, a new triterpenoid alcohol isolated from the barks of several species of the *Genus Ilex*, has now been oxidized with chromium trioxide to yield a ketone, designated as ilexone of $C_{30}H_{48}O$, m.p. 239.5–240.5°, and $[\alpha]_D^{14}-68.74°$, which gives oxime of m.p. 256.5–257.5°, 2,4-dinitrophenylhydrazone of m.p. 260–261°, and positive Zimmermann test.

On reduction with sodium and ethanol as well as with lithium aluminum hydride, it has been shown that ilexone is regenerated to yield an alcohol proved to be identical with natural ilexol.

On reduction according to the procedure of Huang-Minlon, ilexone has been shown to yield an oxygen-free compound, designated as ilexene of $C_{30}H_{50}$, and m.p. 241–242°.

It seems most likely that ilexol might be a triterpenoid alcohol of $C_{30}H_{49}OH$, an alcoholic hydroxyl group of which is secondary in nature, and moreover attached at C-3 in its molecule.

Ilexol of $C_{30}H_{50}O$, m.p. 205–206° and $[\alpha]_D^{14}$ −29.05° (c, 0.860, $CHCl_3$) is a new triterpenoid alcohol, which was first isolated by one of us from the unsaponifiable fraction of commercial "white brid-lime"[1], and later found to be present in the unsaponifiable fraction of bird-lime from the bark of *Ilex integra* Thunberg[2] as well as from that of *I. crenata* Thunberg[3]†.

In order to elucidate the unknown chemical structure and configuration of this substance, a series of reactions have now been carrid out, and the description and discussion of the results of which are the aim of this paper.

Ilexol used in this investigation, has now been isolated in just the same manner as reported previously[1~3], from the barks of *Ilex integra* Thunberg as well as *I. crenata* Thunberg.

The infrared spectrum of ilexol (Fig. 1) resembles that of β-amyrin, even in the finger-print region, suggestive of their close structural interrelationship between them.

In accord with their strcuture, the presence of hydroxyl groups both in ilexol and in β-amyrin are indicated by sharp absorption bands at 3320 cm^{-1} and 3200 cm^{-1} respectively.

Furthermore, in accord with its structure, the infrared spectrum of ilexol acetate of $C_{30}H_{49}\cdot OCOCH_3$, m.p. 290–291°, and $[\alpha]_D^{14}$ −30.47° (c, 0.549, $CHCl_3$) has now been found to exhibit no appreciable absorption band in the 3200–3500 cm^{-1} region, and to exhibit sharp absorption bands at 1726 cm^{-1} and 1247 cm^{-1}, indicative of the absence of free hydroxyl group and the presence of the $-OCOCH_3$ group in its molecule.

Ilexol has now been show to be oxidized with chromium trioxide in acetic acid solution to yield a ketone designated as ilexone of

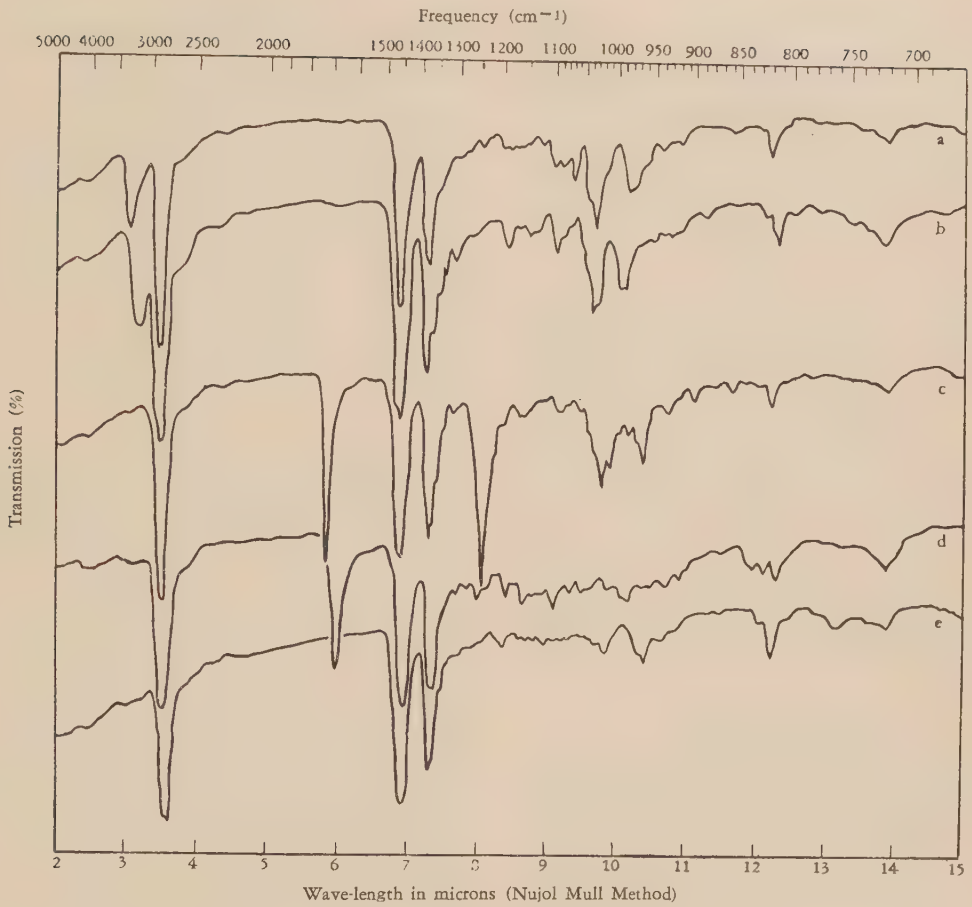
*Ooemachi, Kumamoto City, Japan.

1) S. Iseda, *J. Pharm. Soc. Japan*, **72**, 1064; 1611 (1952).

2) S. Iseda, K. Yagishita, N. Toya, *J. Pharm. Soc. Japan*, **74**, 422 (1954).

3) K. Yagishita, This Bulletin, **21**, 160 (1957).

†A triterpenoid alcohol isolated from the rhizome of *Solidago altissima* Linnaeus (*Compositae*) has recently been shown by T. Takemoto to be none other than ilexol, by mixed m.p. determination as well as by comparison of their infrared spectra. Its acetate has also been shown to be identical with ilexol acetate (mixed m.p. and infrared spectra). (Private communication to one of the authors from Dr. T. Takemoto of Pharmaceutical Department of Osaka University (March 3, 1957)).



a) Ilexol, b) β-Amyrin, c) Ilexol Acetate, d) Ilexone, e) Ilexene.

FIG. 1.

$C_{30}H_{48}O$, m.p. 239.5–240.5°, and $[\alpha]_D^{14}$ −68.74° (c, 0.363, $CHCl_3$), which gives oxime of $C_{30}H_{48}ON$ and m.p. 256.6–257.5° as well as 2,4-dinitrophenylhydrazone of $C_{36}H_{52}O_4N_4$ and m.p. 260–261°, and exhibits positive Zimmermann test[4].

In agreement with this result, the infrared spectrum of ilexone has been shown to exhibit no appreciable absorption band in the 3200–3500 cm^{-1} region, and to exhibit a sharp band at 1690 cm^{-1}, indicative of the absence of hydroxyl group, and the presence of the carbonyl group in its molecule.

On reduction with sodium and ethanol as well as with lithium aluminum hydride, it has been shown that ilexone is regenerated to yield an alcohol proved to be identical with natural ilexol, indicative of the fact that reduction proceeds predominantly to yield only one stereoisomer.

In order to obtain an oxygen-free derivative of ilexol, ilexone has now been subjected to reduction according to the procedure of Huang-Minlon, giving a compound of $C_{30}H_{50}$ and m.p. 241–242°, which would be designated as ilexene hereafter.

4) W. Zimmermann, *Z. physiol. Chem.*, **245**, 47 (1936); D.H. R. Barton, P. de Mayo, *J. Chem. Soc.*, **1954**, 887.

In accord with its structure, the infrared spectrum of ilexene has been shown to exhibit no appreciable bands both in the 3200–3500 cm^{-1} region as well as in the carbonyl group region, but to exhibit a sharp band at 817 cm^{-1}, suggestive of the absence of both hydroxyl and carbonyl groups, and the presence of $>C=C<^{H}$ in its molecule.

All the ultraviolet absorption spectra of ilexol, ilexol acetate, ilexone, and ilexene have now been found to exhibit a maximum absorption band at 240 mμ, a minimum absorption band at 236 mμ, and an inflexion at 250 mμ respectively, indicative of their close structural interrelationship among themselves.

Now it follows from the foregoing results of experiments, that ilexol might be a triterpenoid alcohol of $C_{30}H_{49}OH$, an alcoholic hydroxyl group of which is secondary in nature, and moreover, attached to C-3 in its molecule.

EXPERIMENTAL*

Isolation of Ilexol from the Barks of *Ilex integra* Thunberg and *I. crenate* Thunberg. Ilexol was isolated from the barks of *Ilex integra* Thunberg and *I. crenata* Thunberg in just the same manner as reported previously[2,3]. The dried bark was thoroughly extracted with ether, and the resulting ethereal solution was taken to dryness to yield a tan-colored, viscous mass, which was further dissolved in cold benzene to remove the benzene-insoluble portion. After filtration and removal of benzene, the purified resin was hydrolyzed in the same manner as reported previously with benzene-ethanolic KOH-solution (10%) to yield an unsaponifiable fraction, which was further carefully fractionated to isolate ilexol by way of its acetate, being almost insoluble in ethanol.

The ultraviolet spectra of ilexol: $\lambda_{max.}$ 240 mμ (log ε 3.80); $\lambda_{min.}$ 236 mμ (log ε 3.34); $\lambda_{inf.}$ 250 mμ (log ε 3.13). The ultraviolet spectra of ilexol acetate: $\lambda_{max.}$ 240 mμ (log ε 2.64); $\lambda_{min.}$ 236 mμ (log ε 2.60); $\lambda_{inf.}$ 250 mμ (log ε 2.41).

The infrared spectra of ilexol and its acetate are now shown in Fig. 1a and 1c, respectively.

Oxidation with Chromic Trioxide of Ilexol. To a solution of 500 mg of ilexol in 10 ml of benzene and 70 ml of glacial acetic acid, was added a solution of 49 mg of chromium trioxide in 5 ml of glacial acetic acid at room temperature during 10 min. with vigorous stirring. After being left overnight at room temperature, the resulting reaction mixture was concentrated to a small volume *in vacuo*, diluted with an amount of water, and then thoroughly extracted with ether.

The ethereal layer was thoroughly washed with water, dried with anhydrous Na_2SO_4 and then distilled off. The residue was dissolved in a mixture of petroleum ether and benzene (5 : 2), and the solution was run down through a column of alumina (2 × 6 cm). The column was eluted with 100 ml of the same solvent mixture, giving 420 ml of an eluate, which after removal of the solvent mixture and several recrystallizations from a mixture of chloroform and ethanol (1 : 1), afforded ilexone as prismatic needles, melting at 239.5–240.5°. $[\alpha]_D^{14} -68.74°$ (c, 0.363).

The ultraviolet spectra: $\lambda_{max.}$ 240 mμ (log ε 2.99); $\lambda_{min.}$ 236 mμ (log ε 2.96); $\lambda_{inf.}$ 250 mμ (log ε 2.81). The infrared spectrum is shown in Fig. 1d.

Anal. Found: C, 84.85; H, 11.31. Calcd. for $C_{30}H_{48}O$: C, 84.84; H, 11.39.

Ilexone is sparingly soluble in methanol, soluble in hot ethanol, and readily soluble in acetone, ethyl acetate, chloroform and petroleum ether. W. Zimmermann test[4] for 3-oxo-triterpenoids was found to be positive.

Oxime of Ilexone. To a solution of ilexone (m. p. 239.5–240.5°) in 2 ml of anhydrous pyridine and 3 ml of absolute ethanol, was added 150 mg of hydroxylamine hydrochloride, and the resulting mixture was refluxed for 2 hrs. on a water-bath. After removal of the solvents with aeration, there remained a colorless mass, which was thoroughly washed with 4 ml of cold water, filtered, and then washed with cold water. The solidified residue was recrystallized several times from ethanol to yield colorless fine needles (95 mg), melting at 256.5–257.5°.

Anal. Found: C, 82.01; H, 11.09; N, 3.02. Calcd. for $C_{30}H_{49}ON$: C, 81.94; H, 11.23; N, 3.19.

2,4-Dinitrophenylhydrazone of Ilexone. To a

* Unless stated otherwise, a) all melting points were corrected; b) values of rotation were measured in $CHCl_3$-solutions, using 1 dm tubes; c) ultraviolet spectra were measured in cyclohexane solution with a Beckman DU spectrophotometer; d) infrared spectra were measured in Nujol with a Perkin Elmer 21 double-beam instrument provided with NaCl prisms; and e) chromatographic separation of the column was carried out by using alumina (200 mesh, in size; purchased from Wako Pure Chemicals Co.) as an adsorbent, and petroleum ether (b.p. 60–80°) as an eluant.

solution of 0.2 g of 2,4-dinitrophenylhydrazine in 1 ml of conc. H_2SO_4 was added dropwise 1.5 ml of water with stirring to yield a yellowish solution, to which was further added 5 ml of 95% ethanol.

The resulting solution was poured onto a solution of 70 mg of ilexone in 20 ml of ethanol, and the reaction mixture was allowed to stand at room temperature overnight. There separated yellowish precipitate which was thoroughly extracted with ether, and the ethereal layer was washed successively with dil. Na_2CO_3-solution, and with water, and then dried. After removal of ether, there remained 60 mg of a yellowish mass, which was recrystallized several times from a mixture of ethyl acetate and ethanol (1 : 1) to yield 2,4-dinitrophenylhydrazone (55 mg) as fine needles, melting at 260–261° with sintering at 215°.

Anal. Found: C, 71.52; H, 8.45; N, 9.03. Calcd. for $C_{36}H_{52}N_4O_4$: C, 71.49, H, 8.67; N, 9.26.

Ladenburg's Reduction of Ilexone. To a solution of 100 mg of ilexone in 25 ml of absolute ethanol was added 1 g of sodium in portions, during 10 min., and the resulting reaction mixture was further refluxed for 2 hrs.. After being treated in the usual manner, the reduction product was dissolved in 70 ml of a mixture of petroleum ether and benzene (5 : 2), and the resulting solution was run down through a column of alumina (2×5 cm), and the column was eluted with 100 ml of the same solvent, giving an eluate containing 90mg of a fraction, which after removal of the solvents and several recrystallizations from ethanol afforded colorless fine needles of m.p. 205–206°, undepressed on admixture with an authentic specimen of ilexol. $[\alpha]_D^{14}-27.89°$ (c, 0.860, pyridine).

Anal. Found: C, 84.54; H, 11.58. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

On acetylation with 1 ml each of acetic anhydride and pyridine, 50 mg of this alcohol afforded crude acetate of m.p. 283–288°, which after several recrystallizations from ethyl acetate crystallized as rhombic plates (50 mg) of m.p. 290–291°, undepressed on admixture with an authentic specimen of ilexol acetate[2,3]. $[\alpha]_D^{14}-30.49°$ (c, 0.546).

Anal. Found: C, 81.80; H, 10.92. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

Reduction with Lithium Aluminum Hydride of Ilexone. To a solution of 70 mg of ilexone in 70 ml of dried ether, was added 100 mg of lithium aluminum hydride in portions, and the reaction mixture was refluxed for 4 hrs.. After being left at room temperature overnight, the excess of the reagent was decomposed by addition of a few drops of ethyl acetate, after which the reaction mixture was diluted with water, acidified with dil. H_2SO_4, and worked up as described above. Repeated recrystallizations from ethanol of the product afforded colorless fine needles of m.p. 205–206°, undepressed on admixture with an authentic specimen of ilexol.

Anal. Found: C, 82.64; H, 11.58. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

This alcohol was acetylated with acetic anhydride-pyridine to yield crude acetate, which after several recrystallizations from ethyl acetate crystallized as rhombic plates, and melted at 290–291° either alone or on admixture with an authentic specimen of ilexol acetate.

Reduction of Ilexone according to the Procedure of Huang-Minlon. To a solution of 300 mg of ilexone in 80 ml of ethylene glycol was added 3 ml of hydrazine hydrate (80%), and the resulting mixture was heated under reflux for 1 hr. on an oil bath, after which the reaction mixture was left at room temperature overnight.

After addition of 500 mg of KOH to this mixture, the resulting mixture was further refluxed for 6 hrs., cooled, diluted with water, and then thoroughly extracted with benzene. After removal of benzene, there were obtained elongated plates, which were dissolved in 100 ml of petroleum ether, and the solution was run down through a column of alumina (2×6 cm), and the column was eluted with 200 ml of the same solvent, giving an eluate containing 125 mg of a fraction, which after removal of the solvent and several recrystallizations from a mixture of chloroform and methanol (1 : 1) afforded colorless prisms melting at 241–242°.

The ultraviolet spectra: $\lambda_{max.}$ 240 mμ ($\log\varepsilon$ 2.92), $\lambda_{min.}$ 236 mμ ($\log\varepsilon$ 2.86), and $\lambda_{inf.}$ 250 mμ ($\log\varepsilon$ 2.68). The infrared spectra is shown in Fig. 1e.

Anal. Found: C, 87.92; H, 12.09. Calcd. for $C_{30}H_{50}$: C, 87.73; H, 12.27.

Ilexene is almost insoluble in methanol, sparingly soluble in ethanol, and readily soluble in acetone, ethyl acetate, chloroform, and petroleum ether.

A mixture of this substance (m. p. 241–242°) and ilexone (m. p. 239.5–240.5°) melted at 225–231°, indicative of their non-identity.

Acknowledgements. The author's heartiest thanks are due to Prof. Dr. Y. Oshima of Kyushu University, and to Prof. Dr. E. Sebe of Kumamoto University, for their helpful advice

and encouragement throughout the course of this work. They are also deeply indebted to Messrs. H. Kozuma and K. Kawasaki of Technical Department of Minamata Factory of Shin Nippon Chisso Hiryo and Co., Minamata, Kumamoto, for their courtesy of measuring infrared spectra, to Dr. K. Uyeno of Dojin Pharmaceutical Laboratory, Kumamoto for measuring ultraviolet spectra, and to Mr. E. Aoyagi of Technical Department of Oomuta Factory, Mitsui Kagaku Kogyo Co., Oomuta, Fukuoka, for carrying out elementary analyses.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 279~283, 1957]

Studies on the Biosynthesis of Lignin

Part I. Changes of the Contents of Lignin and other Chemical Constituents with Growth of "Yugao" Fruit (*Lagenaria leucantha* Rosby var. *clavata* Makino[1])

By Isamu Tachi, Akira Hayashi and Akira Sato

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University

Received April, 1, 1957

We have been studying on the genesis of lignin and the changes of some chemical constituents contained in the gourd fruits which were taken in turn in certain periods after flowering.

The results obtained are as follows:

(1) Lignin was at first generated on the fifteenth-day after flowering, and since then increases rapidly and finally occupies the whole fruit. The distributions of peroxidase and phenoloxidase in the tissue of fruit agreed with the lignification, and, it is suggested that these enzymes have relations to the biosynthesis of lignin.

(2) Lignin is mainly synthesized at the tissue of the shell-part of fruit. The content of lignin was only 2.3% on the fifteenth-day but it reached to 32% in hardened fruit. The methoxyl content of lignin was only 2% on the fifteenth-day but it increased along with the increase of lignin reaching to a level of 12% on the fifteenth-day, and to 15.3% after drying. In addition to these changes of lignin, other components except lignin also showed characteristic changes.

(3) In order to investigate the increase of the methoxyl content of lignin further, lignin was oxidized with nitrobenzene in an alkaline medium. Among *p*-hydroxybenzaldehyde, vanillin and syringaldehyde in that oxidative mixture, the former was the richest component in the younger fruit, but the latter two, which were richer in their methoxyl content increased with the growth of fruit, and after ripening vanillin was the richest and *p*-hydroxybenzaldehyde the poorest component.

INTRODUCTION

Recently, much attention has been paid to studies on the biosynthesis of plant lignin[2)~9)], -above all, the results of Freudenberg's works[2)~4)] are bringing epoch-making progresses to the chemistry of lignin.

Shigeno[10)], several years ago found that when a gourd named "yugao" (*Lagenaria leucantha* Rosby var. *clavata* Makino) or "fukube" (*Legenaria leucantha* Rosby var. *depressa* Makino) after a suitable period for the manufacture of dried gourd sharings named "kampyo", a kind of food, was cut off, the tissue under the epidermis became hard showing a colour reaction with phloroglucinol-HCl solution. The authors took interest in this

1) T. Makino, Illustrated Flora of Nippon, p. 89, Hokuryukan, Tokyo (1940).
2) K. Freudenberg, *Fortschritte d. Chemie org. Naturstoffe*, XI S. 43 (1954).
3) K. Freudenberg, *Naturwisss.*, **42**, 29 (1955).
4) K. Freudenberg, *Angew. Chem.*, **69**, 84 (1956).
5) S.M. Manskaja, *Biochimia G.*, **711** (1952). (*Chem. Abst.*, **47**, 5115 (1952).
6) S M. Siegel, *Physiol. Plant.*, **8**, 20 (1955).
7) G. Eberhardt, F.F. Nord, *Archives Biochem*, **55**, 578 (1955).
8) T. Higuchi, I. Kawamura, *J. Japanese Forestry Soc.*, **37**, 502 (1955).
9) H. Ishikawa, *J. Japanese Forestry Soc.*, **37**, 244 (1955).
10) U. Shigeno, *Bull. of the Agr. Utsunomiya Univ.*, **1**, 441 (1950-53).

phenomena because it was thought that a part of the mechanism of lignification in the living cell might be elucidated by a profound investigation of the matter.

The gourd flowers were provided from July to August; from thirty to fifty days after the flowering, the fruit was taken and dried and the seed was gathered. The hardenedshell was used for fancy goods such as charcoal-cases or cups.

In order to conduct a study on the lignification in the gourd tissue, the authors took fruits in turn a certain period after flowering, and with a microscope investigated the formation of lignin and the distribution of peroxidase and phenoloxidase in the gourd tissue. Moreover, the authors investigated the changes of the contents of lignin and other important constituents along with the growth of fruit.

EXPERIMENTAL

I. Samples

The gourds were taken on the twelveth-day, the fifteenth-day, the eighteenth-day, the thirtieth-day and the fiftieth-day after flowering, these being named samples A, B, C, D, and E. The dry hardened tissue which is used for fancy goods was also used as sample F.

II. Microscopic Observations

(1) Lignin Slices of one hundred-μ thickness, cut off from samples A to E were investigated for the distribution of lignin contained in the tissue, by means of the color reaction developed by the dropping of phloroglucinol-HCl solution. These results are shown in Fig. 1.

In the young sample A, no lignin was observed except that of the spiral vessel. The spiral vessel was included in fibrovascular bundle which ran irregularly among the parenchyma, and a faint color was observed by the phloroglucinol-HCl reaction.

In sample B, lignin was found at the cell-wall of the outer mesocarp in contact with hypoderm. In sample C, lignification in the outer mesocarp proceeded pretty well and a weak color reaction was also observed around the fibrovascular bundle. In sample D, the lignified layer expanded all over the outer mesocarp, and in sample E, it anewly expanded in the great spherical cell of the middle mesocarp. At this period, the lignified layer was over one-cm in thickness and the outer shell was fairly hardened. In the hardened sample F, phloroglucinol-HCl reaction was positive in all parts, and the lignification was completed.

(2) Peroxidase Peroxidase distribution in the gourd tissue was investigated by the color reaction developed by the contact of filter papers containing 1% benzidine and 3% hydrogene-peroxide.

In the younger samples, peroxidase was found all over the parenchyma. Above all, at the outer mesocarp and the circumference of the fibrovascular bundle, in which lignin would be formed, peroxidase was found densely.

In the grown samples, however, peroxidase disappeared from the lignified cell.

These facts showed that peroxidase was concerned with lignification. Moreover, no peroxidase could be found at the hypoderm in the younger samples, but it was observed in the grown samples, such as D and E.

(3) Phenoloxidase Phenoloxidase was detected by the color reaction developed with the dropping of N/50 phenylenediamine solution. Consequently, it was found that phenoloxidase in every sample existed at the same position as lignin.

III. Changes of the Contents of the Chemical Constituents in the Gourd Tissue with Growth

Samples A to E were divided into the shell part and fleshy part, respectively. The former consisted of the epicarp, hypoderm and some parts of the outer mesocarp, while the latter consisted of the other parts excluding the placenta and seed.

Each part was analyzed, in respect of the following terms shown in Table I.

At first, the sample of 300 g was extracted with 300 ml water by crushing in a mixer and then filtered. Reducing sugar, aldose, and soluble nitrogen contained in the extract were determined by Bertrand's, hypoiodite oxidation and Kjeldahl's method, respectively. Moreover, sugars in this extract were detected by the paperchromatography using butanol, acetic acid, and water (5 : 1 : 2, upper-layer) as a developer.

The residue was then washed thoroughly with water and weighed after drying at 50°C. Thence, pentosan, lignin, methoxyl in lignin, and insoluble nitrogen of the residue were determined by the following methods i.e., TAPPI[11,12], semimicropyridine[13] and Kjeldahl,

11) TAPPI standard, T 222m-50.
12) TAPPI standard, T 223m-48.
13) Y. Asahina, S. Maeda, *J. Pharm. Soc. Japan*, **50**, 660 (1930)

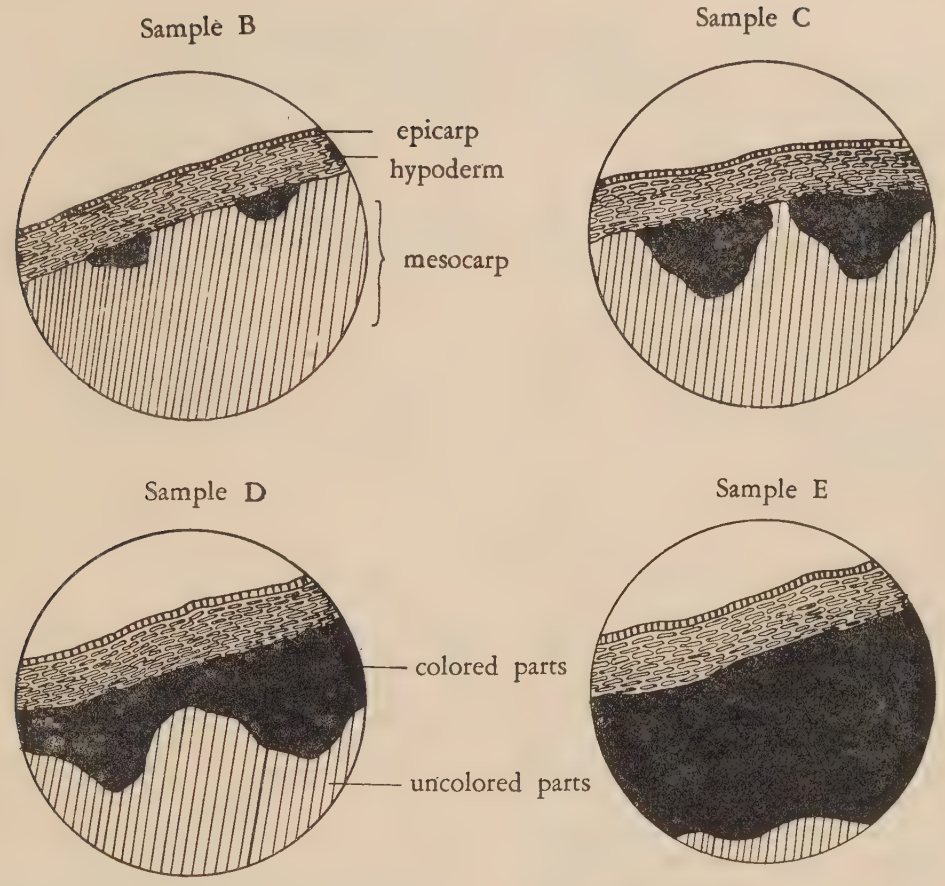


FIG. 1. Distribution of Lignin in Fruit (phloroglucinol-HCl reaction)

TABLE I
ANALYSIS OF "Yugao" (Gourd) FRUIT

Sample (day)	A (12th day)		B (15th day)		C (18th day)		D (30th day)		E (50th day)		F (drying)
Divided Part	Flesh	Shell	Flesh	Shell	Flesh	Shell	Flesh	Shell	Flesh	Shell	Shell
Moisture[a]	94.98	93.92	95.27	94.81	97.15	93.89	96.87	92.61	94.42	91.09	
Water Extract											
Total Extract	65.48	49.75	66.60	48.80	53.17	44.40	70.05	29.47	71.25	18.50	
Reducing Sugar	45.55	37.57	49.97	31.96	48.95	23.82	52.75	17.90	12.90	9.33	
Aldose/Reducing Sugar	69.00	92.11	68.10	95.17	66.27	92.66	87.91	99.10	94.00	98.10	
Soluble Nitrogen	0.44	0.59	0.68	1.39	1.47	0.66	1.50	1.09	1.07	0.80	
Extract Residue											
Total Residue	34.52	50.25	33.40	51.20	46.83	55.60	29.95	70.53	28.75	81.50	
Pentosan (as Xylan)	3.71	5.56	4.75	6.37	6.41	10.64	5.95	10.96	5.39	16.97	19.65
Lignin	1.13	1.44	1.24	4.01	2.44	11.79	1.10	13.32	1.83	23.12	32.58
Methoxyl in Lignin[b]	—	—	1.83	1.87	1.11	7.30	—	8.09	2.35	11.63	15.28
Insoluble Nitrogene	0.49	0.81	0.36	1.28	0.52	0.63	0.35	1.16	0.38	0.45	0.59
Paperchromatography[c]											(Ash) 4.58
Xylose	±	++	±	+	−	+	−	−	−	−	
Fructose	+	+	++	+	++	+	+	±	+	±	
Glucose	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	

(a) Based on Whole Fruit (b) Based on Lignin (c) +++ Very Intense, ++ Intense, +Positive, ± Trace, − Negative.

respectively. The hardened sample F, was analyzed only a series of water insoluble matter.

These results are shown in Table I.

As shown in this table, lignification occurred mainly in the shell part and scarcely in the fleshy part. Also lignification began on the fifteenth-day after flowering at first, proceeding suddenly since that time.

These increases of lignin agreed with the results of the microscopic observation, and the lignin content reached to more than 30% after hardening.

As lignin was generated, considerable changes in other constituents were also observed. For instance, moisture in the fruit, especially in the shell, was decreased, and the water extract in shell was suddenly decreased while in the flesh it increased. Reducing sugar in the water extract which occupied about a half of dry matter in the younger fruits, was decreased vanishing after complete hardening. In the reducing sugars, glucose, fructose and xylose were detected by paperchromatography, glucose being the richest among them. An appreciable amount of fructose was found in the flesh but it was decreased with growth. This relation was also shown by the increase of the ratio of aldose to reducing sugar with growth. Xylose was richer in the shell than in the flesh and pentosan was also richer in the shell.

The methoxyl content in the lignin from the flesh was always only on the level of a few present, so it was doubtful whether this was true in case of lignin. The lignin in the flesh itself, was on the level of only a few percent. However, the methoxyl content in the lignin from the shell was increased with the increase of lignin reaching to a level of 15.3%, similar to ordinary plant lignin. The nitrogen content was increased when changes of other constituents were great, i.e., when the activities of enzymes were vigorous.

IV. Syringaldehyde, Vanillin and *p*-Hydroxybenzaldehyde from Oxidation Product of Lignin by Alkaline Nitrobenzene

A meal of the shell part (30 to 60 mesh), containing lignin of 0.5 g was heated at 160°C in a rotary autoclave with 3 ml nitrobenzene and 50 ml 2N sodium hydroxide. After 2.5 hrs. heating, the oxidized mixture was filtered and neutralized by acetic acid to pH 5.

Three aromatic aldehydes were separated from this oxidized solution by the paperchromatographic method.

The oxidized solution was spotted on paper ("Toyo" No. 50, filter paper) and after drying a small quantity of 2% HCl solution was sprayed on the whole paper. Then this paper was developed by benzene, ligroin (b.p., 100 to 120C°), methanol and water (50 : 50 : 1 : 50, upper-layer). By this method, though the R_F values of the three aldehydes were slightly changed

with the amount of sprayed HCl, the separation of aldehydes was evident. The mean R_F-values of vanillin, syringaldehyde and *p*-hydroxybenzaldehyde developed by 2,4-dinitrophenylhydrazine, were 0.60, 0.43 and 0.09, respectively. These were confirmed by comparison with authentic samples and ultraviolet absorption in an alkaline alcoholic solution.

The separated aldehydes were determined with ultraviolet absorption by Lemon's method[14], and the results are shown in Table II, in which the relation among the three aldehydes are shown by their molar ratio and percentage.

Table II
Molar Ratio among Three Aldehydes

Sample	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde
B	1.0 (54%)	0.60 (32%)	0.26 (14%)
C	1.0 (42%)	1.03 (44%)	0.34 (14%)
D	1.0 (38%)	1.21 (46%)	0.43 (16%)
E	1.0 (26%)	2.20 (56%)	0.70 (18%)
F	1.0 (13%)	4.70 (61%)	2.00 (26%)

As shown in this table, *p*-hydroxybenzaldehyde was decreased with growth, but vanillin and syringaldehyde which were richer in methoxyl content than *p*-hydroxybenzaldehyde were increased. These results agreed with the increasing of methoxyl content in lignin with growth.

DISCUSSION

As these experimental date show, the gourd, "yugao", fruit contains no lignin during a certain period though the fruit itself grows fairly, but thereafter lignification proceedes suddenly in the tissue. This lignification of the fruit, however, is not generally common in *Cucurbitaceae* occurring only in several species, such as "yugao". "Yugao" fruit is thus an interesting object for conducting studies on biosynthesis of lignin.

According to Freudenberg[3], in softwood, the precursor of lignin is coniferylalcohol, which is polymerized by the action of oxidizing enzyme "redoxase". Moreover, according to Siegel[6], Higuchi[8], and Ishikawa[9], peroxidase and phenoloxidase act as catalysts in case of lignification.

From the results of this investigation, the distributions of peroxidase and phenoloxidase also show close relations to lignification. Especially, for peroxidase which is abundant in the tissue which would be lignified, and disappears gradually from the lignified tissue.

Since lignin is mainly formed in the outer mesocarp inside the green hypoderm, it is suggested that the formation of lignin will have relation to chlorophyll. Moreover, since phloroglucinol-HCl reaction occures in the vessel in the first place, it is probable that lignin precursors (coniferylalcohol and sinapylalcohol by Freudenberg[3], *p*-hydroxycinnamylalcohol by Nord and Eberhalt[7]) result from other parts, such as the stalk and the leaf, via the vessel. However, it is unknown whether the lignin precursor is synthesized in the fruit or results from the other parts.

The methoxyl content in liginin increases with growth, and its content in grown fruit reaches to a level of 15.3%, similar to other plant-lignin. This increase agrees with the relations among *p*-hydroxybenzaldehyde, vanillin and syringaldehyde from oxidative products of lignin. It seems that these facts support Nord's hypothes[7], of which lignin is formed by the transmethylation of the polymer of cinnamylalcohol type precursor.

14) H. W. Lemon, *Anal, Chem.*, **19**, 847 (1947).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 284~290, 1957]

Studies on the Amylolytic System of the Black-koji Molds

Part II. Raw Starch Digestibility of the Saccharogenic Amylase Fraction and its Interaction with the Dextrinogenic Amylase Fraction

By Seinosuke Ueda

Laboratory of Zymology and Industrial Fermentation, Department of Agriculture, Kyushu University, Fukuoka, Japan

Received April 5, 1957

The amylase system of black-koji molds was fractionated, in respect to its activity, into two fractions, the dextrinogenic and saccharogenic. Their separate and combined activities to digest raw starch were investigated. Contrary to the knowledge established so far, the dextrinogenic amylase fraction was found to be capable of digesting the raw starch though only weakly, while the saccharogenic amylase fraction much more strongly. When the two fractions were combined, digestion proceeded with twice or thrice the velocity of the sum of each component. This accelerating interaction, which never occurs in cooked-starch digestion, is worthy to be stressed as a characteristic of raw starch digestion by this amylase system. The raw starches of corn, wheat and glutinous rice are in this way, digested completely to glucose. Among them, glutinous rice starch displays peculiar facility in the digestion.

INTRODUCTION

In the previous report, Yamasaki and the author[1] have found that the digesting action of black-koji amylase system on raw starch is much stronger than that of yellow-koji and malt-amylase. After dividing this system into dextrinogenic and saccharogenic amylase fractions, the author[2] has recently investigated the amylolytic properties of this system through digesting-experiments on cooked-starch, amylopectin, glycogen and β-limit dextrin, and adsorption-experiments on both fractions onto raw corn starch. From the latter experiments, the "debranching-factor" was revealed to be adsorbable on raw corn starch, most readily at pH 3.6, the value near the optimal pH 3.8 for the amylolytic activity of the system.

Now, on the basis of these results, the raw starch digestion was treated in the present paper, in almost the same experimental line as that of cooked-starch.

By using germinated wheat amylase, Stamberg and Bailey[3] showed that raw wheat starch was digested by α-amylase up to 4–10 per cent, and by β-amylase to only 1 per cent hydrolysis. Blish, Sandstedt and Mecham[4] reported that wheat might contain an enzymatic "raw starch factor", which is responsible for raw starch digestion, different from α-amylase. Balls and Schwimmer[5] found a high activity of raw starch digestion in a mixture of pancreas extract and mold bran extract. Schwimmer[6], in his report suggested that maltase, in addition to the recognized α-amylase, participates a part in the rapid degradation of raw starch. Sandstedt and Gates[7] found that, of the four α-amylase

1) I. Yamasaki and S. Ueda, *J. Agr. Chem. Soc. (Japan)*, **24**, 181 (1951).

2) S. Ueda, This Bulletin, **20**, 148 (1956).

3) O.E. Stamberg and C.H. Bailey, *Cereal Chem.*, **16**, 319 (1939).

4) M.J. Blish, R.M. Sandstedt and D.K. Mecham, *Cereal Chem.*, **14**, 605 (1937).

5) A.K. Balls and S. Schwimmer, *J. Biol. Chem.*, **156**, 203 (1944).

6) S. Schwimmer, *J. Biol. Chem.*, **161**, 219 (1945).

7) R.M. Sandstedt and R.L. Gates, *Food Research*, **19**, 190 (1954).

systems from different sources, a wide variation in the digesting activity of raw starch exists, and also that a cationic detergent "Roccal", when added, in some cases, has effect on the digesting action. In general, from the reports of the above mentioned workers it has been shown that α-amylase will be the principal agent responsible for raw starch digestion.

MATERIALS AND EXPERIMENTAL

Enzyme Preparations A Kawachi strain of *Aspergillus awamori*, the most popular "black-koji" mold, was mostly used in our work.

Saccharogenic amylase fraction (SAF). As described in the previous report[2], this was prepared by acid treatment of black-koji mold bran extract. This amylase fraction also possessed considerable maltase activity.

Dextrinogenic amylase fraction (DAF). Dextrinogenic activity was less sensitive to alkali than the accompanying saccharogenic activity in the black-koji amylase system. Black-koji mold bran extract, prepared as described in the previous report[2], was brought to pH 7.8 with 1 M sodium citrate and held for 15 minutes at 60°C followed by cooling, neutralization and filtration. Then the filtrate was concentrated in vacuum at 38°C to one-tenth of the initial volume. This concentrated solution, dialyzed once again overnight against tap water under toluene, was used as the enzyme solution of dextrinogenic amylase fraction. This fraction possessed no maltase activity. Another dextrinogenic amylase preparation was also prepared from the shaking culture of *Aspergillus usamii* (another species of the black-koji mold) as described in the previous report[2] and this amylase fraction possessed maltase activity.

SAF as well as DAF prepared above was still quite impure and became considerably weak in activity in the process of fractionation. In order to raise the activity to a level of the initial enzyme extract, both of them were used in experiments after being concentrated in vacuum up from one-tenth to one-twentieth.

Determination of Enzyme Activities Enzyme activities were determined and represented in units, as described in the previous report[2].

Adsorption of Enzyme Solution Corn starch adsorption was applied to the amylase fraction in the same procedure as previously reported[2].

Substrates The raw starch substrates were samples of commercial corn starch and wheat starch, and glutinous rice starch prepared in this laboratory.

Determination of Raw Starch Digestion The method used to determine raw starch digestion was the same as previously described by the authors[1]. Essentially, the method consists of a digestion at 30°C with occassional stirring of 0.25 g of raw starch in 40 ml of a solution containing the enzyme and buffer (pH 3.6) in the presence of toluene. At suitable time-intervals, the reducing sugar formed in the reaction mixture was determined by micro Bertrand's method, hence, the degree of hydrolysis was calculated in terms of per cent of the glucose formed on the theoretical glucose value of starch. It is only in the case of DAF without maltase activity that reducing sugar is calculated as maltose.

Since raw starch digestion, in general, takes a relatively long time of about 60, or sometimes 160 hours to reach a recognizable extent of hydrolysis, one must be aware of the stability of enzyme activity in experimental duration. A preliminary test showed no decrease in both activities during the 7-day incubation, under the above conditions.

Paper Chromatography of Reducing Sugars The procedure described in the previous report[2] was used. Namely, this was carried out by the multiple ascendent technique using a *n*-butanol-pyridine-water mixture (6 : 4 : 3 by volume), and the finished chromatogram was sprayed with aniline phthalate reagent and heated at 120°C for a few minutes. The areas corresponding to reducing sugars appeared in brown spots on the paper.

RESULTS

Activities of Enzyme Solution Dextrinogenic, saccharogenic and maltasic activities were determined and are shown in Table I.

Single and Combined Actions of Dextrinogenic and Saccharogenic Amylase Fractions on Raw Corn Starch The composition of reaction mixtures is shown in Table II, where each of the two single enzyme digests was supplemented with the other enzyme fraction in a heat-destroyed form, so that impurities in the reaction mixture might be equal, in all the three digests. As shown in Figs. 1 and 2, the rate and extent of raw starch digestion, traced in hydrolysis-hour curves, of these three

TABLE I
ACTIVITIES OF VARIOUS ENZYME SOLUTIONS

Enzyme Solution	Origin	Activities (units) Dextrinogenic	Saccharogenic	Maltasic
Dextrinogenic Amylase Fraction-I	mold bran	1.9	(1.20)	0
Dextrinogenic Amylase Fraction-II	shaking culture*	3.3	(1.82)	1.00
Saccharogenic Amylase Fraction-I	mold bran	0	2.02	1.20
Saccharogenic Amylase Fraction-II	mold bran	0	2.99	1.86

*Dextrinogenic Amylase Fraction-II was prepared from shaking culture of *Aspergillus usamii*.

TABLE II
COMPOSITION OF REACTION MIXTURES

No.			1	2	3
Dextrinogenic Amylase Fraction		ml	10	0	10
Saccharogenic Amylase Fraction		ml	0	10	10
Heat-Inactivated	Dextrinogenic Amylase Fraction	ml	0	10	0
	Saccharogenic Amylase Fraction	ml	10	0	0
Buffer Solution (M/10 citrate-HCl, pH 3.6)		ml	10	10	10
Distilled Water		ml	10	10	10
Total Volume		ml	40	40	40
Raw Corn Starch		g	0.25	0.25	0.25

added toluene 0.5ml.

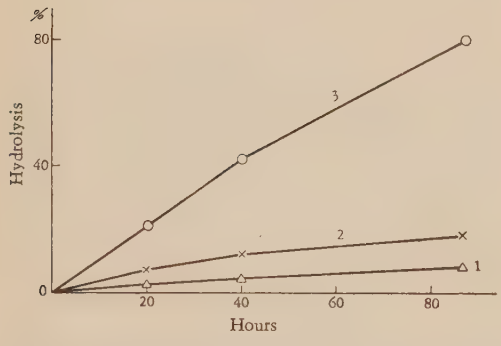


FIG. 1. Single and Combined Actions of Dextrinogenic and Saccharogenic Amylase Fractions on Raw Corn Starch.

Curve 1, dextrinogenic amylase fraction-I alone (—△——△—).
Curve 2, saccharogenic amylase fraction-I alone (- - ×——×—).
Curve 3, dextrinogenic -I and saccharogenic -I amylase fractions combined (—○——○—).

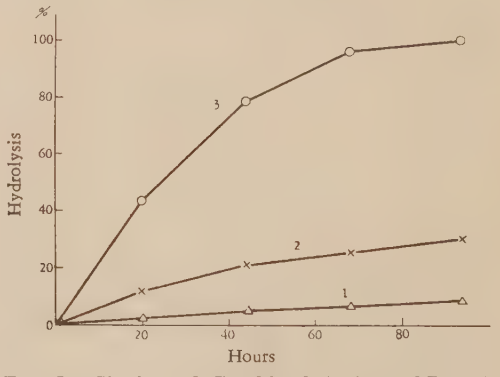


FIG. 2. Single and Combined Actions of Dextrinogenic and Saccharogenic Amylase Fractions on Raw Corn Starch.

Curve 1, dextrinogenic amylase fraction-II alone (—△——△—).
Curve 2, saccharogenic amylase fraction-II alone (—×——×—).
Curve 3, dextrinogenic -II and saccharogenic -II amylase fractions combined (—○——○—).

digests represented very distinct and characteristic patterns. DAF alone (curve 1), had a scanty activity, regardless of the presence or absence of maltase activity and digested only up to 10 per cent even after a 90-hours' incubation. SAF alone (curve 2), on the contrary, displayed moderate activity and was 2 to 4 times as strong as DAF, in the whole course of digestion, but the extent of digestion could not yet exceed 30 per cent hydrolysis in 90 hours. When these two fractions used were combined (curve 3), the digestion proceeded very smoothly, reaching an extent of 80 per cent hydrolysis with almost linear velocity in either 50 or 90 hours, up to the complete digestion in 90

hours (Fig. 2). Thus, the raw starch-digesting activity, in this case, increased by interaction of the two amylase fractions up to about 3 times the sum of each single enzyme activity. Paper chromatographic analysis showed that glucose was the only sugar produced in these digestions, which is highly natural because of the presence of more or less maltasic activity in each digest.

Single and Combined Actions of the Dextrinogenic and Saccharogenic Amylase Fractions on Raw Wheat Starch and Glutinous Rice Starch

As illustrated in Fig. 3, susceptibility of raw wheat starch to each single and combined amylolytic enzymes was almost the same as that of raw corn starch.

Raw glutinous rice starch, however, as shown in Fig. 4, gave an unique pattern of the digestion-hour curves different from that of raw corn and wheat starch. It is a remarkable fact that raw glutinous rice starch could be easily digested by single SAF and completely hydrolysed at least, in a period of 160 hours without any participation of DAF. In this case, the complementary effect of DAF on the amylolysis by SAF, however, was clearly shown in almost the whole range of the digestion, especially vividly in the earlier-stage, but faded off gradually as digestion progressed. On the other hand, resistance of glutinous rice starch toward DAF appeared to be just the same as other raw starches.

Action of Saccharogenic Amylase Fraction on

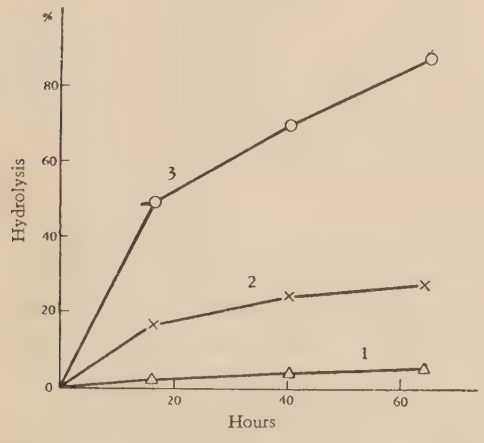


FIG. 3. Single and Combined Actions of Dextrinogenic and Saccharogenic Amylase Fractions on Raw Wheat Starch.

Curve 1, dextrinogenic amylase fraction -II alone (—△——△—).
Curve 2, saccharogenic amylase fraction -II alone (—×—— ×—).
Curve 3, dextrinogenic -II and saccharogenic -II amylase fractions combined (—○——○—).

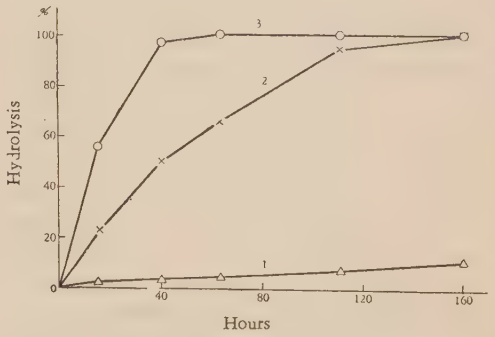


FIG. 4. Single and Combined Actions of Dextrinogenic and Saccharogenic Amylase Fractions on Raw Glutinous Rice Starch.

Curve 1, dextrinogenic amylase fraction-II alone (—△——△—).
Curve 2, saccharogenic amylase fraction-II alone (—×——×—).
Curve 3, dextrinogenic -II and saccharogenic -II amylase fractions combined (—○ —○—).

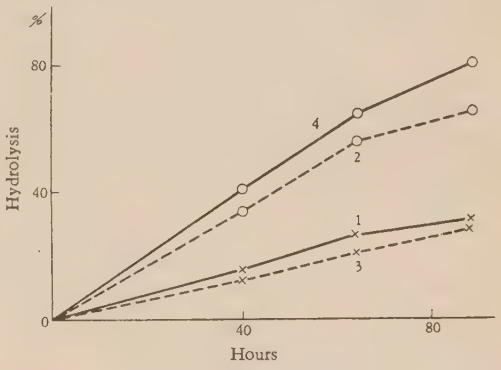


FIG. 5. Action of Saccharogenic Amylase Fraction on the Raw Starch preliminary affected by Dextrinogenic Amylase Fraction.

Curve 1, saccharogenic amylase fraction-II alone on intact corn starch (—×——×—).
Curve 2, saccharogenic amylase fraction-II on the treated and acidic washed (pH 3.6) corn starch (··○····○··).
Curve 3, saccharogenic amylase fraction-II on the treated and non-acidic washed (pH 6.8) corn starch (··×····×··).
Curve 4, dextrinogenic and saccharogenic amylase fractions combined on intact corn starch (—○——○—).

the Raw Starch Preliminary Affected by Dextrinogenic Amylase Fraction At first, two portions of raw corn starch were preliminary digested with DAF, up to about 10 per cent hydrolysis. The starch of each portion treated in this way was collected by centrifugation and washed respectively with acidic (pH 3.6) and non-acidic (pH 6.8) citrate buffer solutions (0.1 M). These two pretreated raw starches were then put under the action of SAF (Fig. 5). In parallel with these two, the digestions of intact raw starch were also carried out as control experiments, by SAF alone and SAF with DAF combined. As shown in Fig. 5, it is an interesting fact that the complementary effective principle in DAF, most probably the dextrinogenic amylase itself, was found to be adsorbable onto corn starch and to be elutable from it into pH 6.8-buffer, whereas, with an acidic buffer (pH 3.6) it could never be eluted.

The Relationship between the "Debranching-Factor" and the Raw Starch Digestion As reported previously[2], SAF, when treated with corn starch adsorption, became much less active in hydrolysing β-limit dextrin from glycogen than before and this was assumed to be attributable to the loss of a "debraching-factor" by adsorption.

Now, in order to find out the relationship between the "debranching activity" and the ability of raw starch digestion, SAF, which was made inactive in debranching action by repeated corn starch adsorption, was used in combination with DAF in raw corn starch digestion. Enzymatic activities of the preparation are shown in Table III. In a comparative experiment, the initial SAF was also used. The composition of reaction mixtures is given in Table IV. In order to level the units of saccharogenic activity, the treated SAF, was used 4 times in volume (10 ml) as large as the initial fraction (2.5 ml).

Comparing the two digest-series in Fig. 6, e. g. curves 1, 2 and 5, and curves, 1, 3 and 4, two important points became clear: (1) The above described accelerating interaction between SAF and DAF disappeared after the adsorption of the responsible principle on corn starch. (compare curve 5 with curve 4); (2) Despite having the same saccharifying activity units measured toward cooked soluble starch, the treated SAF acted only one-fourth as weak as the initial in raw starch digestion (compare curve 2 with curve 3). These results suggest

TABLE III
EFFECT OF ADSORPTION TREATMENT* WITH CORN STARCH

Activities	Saccharogenic Activities		Maltasic Activities	
	units	ratio	units	ratio
Before Treatment	2.77	100	1.52	100
After Treatment	0.72	26	0.40	26

*Adsorption treatment was repeated four times.

TABLE IV
COMPOSITION OF REACTION MIXTURES

No.			1	2	3	4	5
Dextrinogenic Amylase Fraction		ml	2.5	0	0	2.5	2.5
Initial Saccharogenic Amylase Fraction		ml	0	2.5	0	0	2.5
Adsorption-treated Saccharogenic Amylase Fraction		ml	0	0	10	10	0
Heat-Inactivated 100°C, 30 min.	Dextrinogenic Amylase Fraction	ml	0	2.5	2.5	0	0
	Saccharogenic Amylase Fraction	ml	2.5	0	2.5	2.5	0
	Treated Saccharogenic Amylase Fraction	ml	10	10	0	0	10
Buffer Solution (M/10 citrate-HCl, pH 3.6)		ml	10	10	10	10	10
Distilled Water		ml	15	15	15	15	15
Total Volume		ml	40	40	40	40	40
Raw Corn Starch		g	0.25	0.25	0.25	0.25	0.25

added toluene 0.5ml.

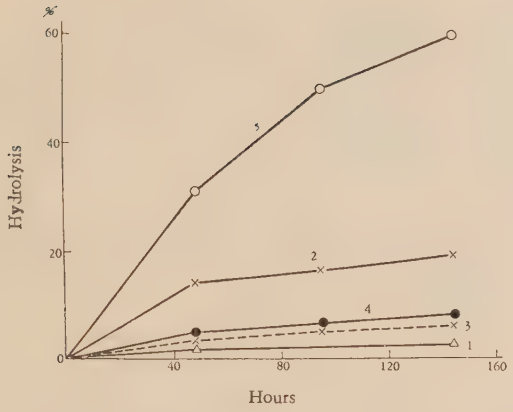


FIG. 6. Effect of Corn Starch Adsorption of Saccharogenic Amylase Fraction on Raw Starch Digestion.

Curve 1, dextrinogenic amylase fraction-II alone (—△——△—).
Curve 2, initial saccharogenic amylase fraction alone (—×——×—).
Curve 3, adsorption-treated saccharogenic amylase fraction alone (··×····×··).
Curve 4, dextrinogenic and adsorption-treated saccharogenic amylase fractions combined (—●——●—).
Curve 5, dextrinogenic and initial saccharogenic amylase fractions combined (—○——○—).

that a "debranching active amylase" in SAF which can be adsorbed on to corn starch must play the key role in raw starch digestion.

DISCUSSION

It is a striking property concerning raw starch digestion that SAF and DAF, when jointly used, interact in such a way that affects the promotion of digesting activity, up to about 3-times the sum of the activity of each fraction.

This fact had hitherto never been observed in the amylolysis of cooked or raw starch with any amylase system of other sources.

In this interacting reaction, SAF will be the essential agent and DAF the accelerating one, because in the digestion of raw glutinous rice starch the SAF was singly able to saccharify the starch completely although a relatively long time of 160 hours was required for this as compared with 60 hours spent in the joint reaction with DAF as shown in Fig. 4. This particular easiness in digesting glutinous rice starch with SAF alone was markedly manifested, as reported previously, in cooked-starch digestion, also. The reason why glutinous rice starch shows this specific property in chemical point of view presents an interesting thesis for conducting studies on the construction of starch granules.

On the mechanism of this accelerating interaction, nothing is described so far.

An accelerating effect of mold maltase to the raw starch digestion by pancreatic extract, on which Schwimmer reported[6], has, of course, no relation with the characteristic interacting effect of this report.

In the previous report[2], the primacy of SAF in the amylolysis was already shown in the digestion of cooked-glutinous rice starch and glycogen.

The SAF, however, could be deprived of its response to the DAF, when its 1,6-glucosidic linkage-degrading activity was taken off by corn starch adsorption (Fig. 6). From these results it is concluded that an unknown substance probably of enzymatic nature and most possibly of an amylolytic one, which develops the "debranching activity" in the SAF is the supreme principle of this characteristic and raw starch digestible amylase system of black-koji mold.

Another noticable fact is that the active principle of DAF can be adsorbed and kept on raw corn starch, optimally at pH 3.6, and can be eluted from the adsorbate at pH 6.8. And the principle of DAF once adsorbed on raw corn starch, reacts in the condition as it is with SAF, which is added afterwards, fulfilling the interacting reaction in digesting the corn starch.

SUMMARY

In concern of the amylase system of black-koji mold, the following facts are described:

(1) The dextrinogenic amylase fraction has an extremely weak action on raw starch, independent of the presence or absence of maltase activity, as compared with the saccharogenic amylase fraction.

(2) The saccharogenic and dextrinogenic amylase fractions, when used combined, interact each other, to promote digestion of raw starch up to about 3-times the sum of activity of each fraction.

(3) This accelerating-interaction effect solely depends upon the "debranching activity" of saccharogenic amylase fraction.

(4) Corn, wheat and glutinous rice starches in a raw state are able to be digested to completeness with this combined system.

(5) Glutinous rice starch shows peculiar easiness in digestion either by saccharogenic amylase fraction alone or by the interacting system combined with the dextrinogenic amylase fraction.

The author wishes to thank Prof. I. Yamasaki for his encouragement and helpful discussion of the problem.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 291~295, 1957]

Studies on Amino Acids. V.

Studies on the Enzymatic Resolution (IV). Enzymatic Resolution of DL-Methionine (2)*

By Ichiro CHIBATA, Akira WATANABE and Shun'ichi YAMADA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

Received April 10, 1957

The enzymatic resolution of acetyl-DL-methonine previously reported was studied further, in detail. As a result, it was found that metal ion plays an important role on the asymmetric hydrolysis of acyl-DL-methonines by the enzyme preparations of *Aspergillus oryzae*.

In the previous paper[1], the authors reported the enzymatic resolution of DL-methionine through the asymmetric hydrolysis of acetyl-DL-methionine by the enzyme preparations obtained from *Aspergillus oryzae* and pancreas. For the purpose to obtain further advantageous conditions of the resolution process, a study on the more detail of the enzymatic reaction was carried out and is presented in this paper. Especially, the influence of metal ion toward the enzme prepared from *Aspergillus oryzae* was investigated and compared with that of renal acylase.

MATERIALS AND METHODS

The substrate, acetyl-DL-methionine was prepared by acetylation of DL-methionine with acetic anhydride in acetic acid, and chloroacetyl-L-methionine was prepared by the Schotten-Baumann reaction.

The extent of the enzymatic hydrolysis was determined by measurement of the liberated amino acid with the ninhydrin method or the van Slyke procedure. For the comparison of enzyme activity, the measurement was carried out when the susceptible substrate was from 10 to 35 per cent hydrolyzed.

EXPERIMENT AND RESULTS

"Influence of Metal Ions"

1) Effect to the Pancreas Enzyme In the previous paper, the authors reported that pancreatic enzyme preparations such as pancreatin and prot-amylase could hydrolyze acetyl-DL-methionine, asymmetrically. In this enzymatic hydrolysis, the influence of metal ions was investigated by the addition of metal chlorides to the reaction mixture and the results are listed in Table I.

As shown in the Table, Zn^{++} showed apparent inhibitory effect and Mn^{++} was also slightly inhibitory. There was no ion capable of accelerating the enzyme reaction significantly.

2) Effect to the Aspergillus *Enzyme* (*Mold Acylase*) Effect of metal ions towards the hydrolytic action of the enzyme produced by the *Aspergillus oryzae* such as Taka-diastase extract and the partially purified preparation, was investigated with the addition of metal chlorides. The partially purified preparation employed for this study was prepared as follows: The extract of Taka-diastase was brought to 0.6 saturation with ammonium sulfate at pH 6.0 and centrifuged. The precipitate was dissolved in water and dialized for 70 hours against cold flowing water. To the dialized solution acetone was added (60% by volume) at-3~-4° and the precipitate was collected and dried.

TABLE I.
EFFECT OF METAL IONS ON THE HYDROLYSIS OF ACETYL-DL-METHIONINE BY PANCREATIN+

	Activity in the Presence of the Ions							
Metal	None	Cu	Mg	Ca	Zn	Mn	Co	Ni
Activity++	100	108	111	114	48	75	105	111

+A solution containing 0.1M acetyl-DL-methionine, pancreatin extract and 10^{-3}M metal chloride was incubated at 37° and pH 6.8, for 24 hours.
++Activity expressed as per cent of control.

*Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Kyoto, April 21, 1956.
**Honjo-Kawasaki-cho, Oyodo-ku, Osaka
1) S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Jap.*, **75**, 113 (1955).

TABLE II
EFFECT OF METAL IONS ON THE HYDROLYSIS BY *ASPERGILLUS* ENZYME

Exp. No.	Enzyme	Activity in Presence of the Ions								
		None	Cu	Mg	Ca	Zn	Cd	Mn	Co	Ni
1*	Taka-diastase extract	100	9	101	80	16	27	29	181	71
2**	Partially purified prep.	100	0	100	97	39	19	85	150	82

*A solution containing 0.1M acetyl-DL-methionine, Taka-diastase extract and 10^{-3}M metal chloride was incubated at 37° and pH 6.8 for 24 hours.
**A mixture of 1ml of 0.05M acetyl-DL-mithionine, 1ml of the enzyme solution, metal chloride (10^{-3} final concentration) and 1ml of Veronal buffer was incubated at 37° and pH 7.0, for 1.5 hours.

This powdered preparation, about 10-fold purified, was dissolved in water and used for the experiment.

The results of the study, which are shown in Table II, disclosed that Cu^{++}, Cd^{++} and Zn^{++} were inhibitory with both preparations. Although Mn^{++} was apparently inhibitory in prolonged incubation with the crude extract, (Table II, exp. 1), the ion showed no decrease of activity under the conditions employed in exp. 2. Besides exp. 1, shown in the Table, another experiment employing phosphate buffer was also carried out, and the only difference observed was the lack of inhibitory effect of Zn^{++}, while, activation by the addition of Co^{++} was evident when either the crude extract or dialized preparation was employed in this enzymatic hydrolysis.

"Inactivation by EDTA and Reactivation by Metal Ions"

1) Effect of EDTA By the above described experiment, it was disclosed that Co^{++} enhance the hydrolytic action of the enzyme produced by *Aspergillus oryzae*. So, in order to find out whether Co^{++} is the mere activator or the enzyme requires essentially the metal ion for the hydrolytic action, the effect of metal chealating agent, EDTA, was investigated.

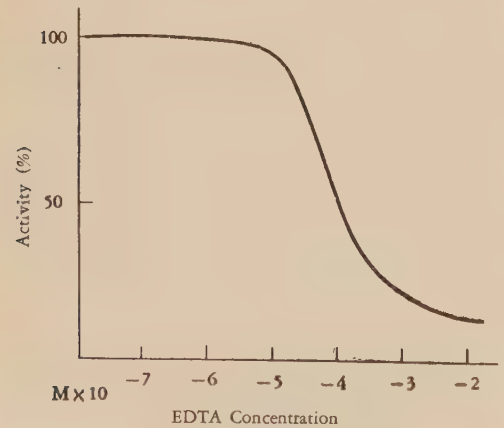


FIG. 1. Effect of EDTA on the Enzymatic Hydrolysis.

To the dialized extract of Taka-diastase treated with various concentrations of EDTA, 0.4M neutralized acetyl-DL-methionine was added and incubated at 37° and pH 6.8, for 2.5 hours. Activity of the enzyme solution without EDTA treatment, taken as 100.

Namely, the extract of Taka-diastase was dialized against cold water for 2 days. No decrease of activity of the enzyme was observed during the dialysis. The dialized enzyme solution was treated with EDTA in concentrations ranging from 10^{-2} to 10^{-8}M, at 0° and pH 7 for 1 hour, then hydrolytic activity was determined. As shown in Fig. 1, the enzyme was markedly inactivated from the 10^{-4}M concentration of EDTA.

2) Reactivation by Metal Ions For further study of the relation between the enzyme action and metal ion, along with the experiment on the effect of metal ions to the partially purified preparation of the *Aspergillus* enzyme described in the previous chapter, the experiment was simultaneously carried out with the EDTA treated enzyme so as to investigate the reactivation by metal ions. The enzyme solution was treated with a 10^{-3}M concentration of EDTA at room temperature and pH 7 for 1 hour, then dialized against cold distilled water for 2 days. This solution was preincubated with metal chloride and activity was determined. The activity was represented as per cent of control without EDTA treatment for comparison and is shown in Table III.

The treatment with EDTA abolished almost completely the enzymatic hydrolysis of acetyl-DL-methionine. Although this inhibition could be reversed only partially, by the addition of Zn^{++} and other ions, reactivation by Co^{++} proved to be most remarkable among the metal ions investigated. (When reactivated with Co^{++}, the EDTA treated-enzyme showed activity even higher than that of those treated. This may be interpreted that the inhibitory substance of the preparation is removed by EDTA treatment and activation of Co^{++} becomes more effective).

TABLE III
REACTIVATION OF INACTIVATED ENZYME BY METAL IONS

EDTA treatment	Activity in the Presence of the Ions								
	None	Cu	Mg	Ca	Zn	Cd	Mn	Co	Ni
None	100	0	104	97	45	19	100	146	86
Treated	5	0	9	19	60	25	7	176	46

A mixture of 1ml of enzyme solution, metal chloride (final concentration, 10^{-3}M) and 1ml of Veronal buffer was pre-incubated at 37°, for 1 hour. Then 1ml of 0.05M acetyl-DL-methionine was added and incubated at 37° and pH 7.0, for 1.5 hours.

These results suggest that the enzyme is a metal enzyme, that is, the metal ion is not separated by dialysis, however, the enzyme is apparently inactivated by EDTA and reactivated by Co^{++}. Although the decision as to whether or not Co^{++} is an actual component of the enzyme must necessarily wait, until the enzyme has been isolated in the pure form.

"Activation by Cobalt and its Practical Application" The fact that Co^{++} activates the enzymatic hydrolysis of acetyl-DL-methionine, should be applied advantageously in the practical process of enzymatic resolution of DL-methionine. So, studies on the optimal concentration of Co^{++} and its practical resolution in the presence of metal ion were carried out.

1) Effect of Cobalt Ion Concentration The effect of various concentrations of Co^{++} on the enzymatic hydrolysis of acetyl-DL-methionine were investigated. The result, shown in Fig. 2, indicated that the optimal concentration for activation was between 10^{-4}M to 10^{-3}, concentration higher than 10^{-2}M decreased activation under the conditions employed by the authors. However, it may be possible that the optimal concentration for the activation somewhat various according to the purity and concentration of the enzyme employed.

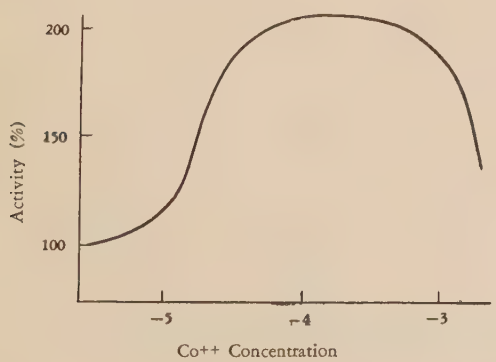


FIG. 2. Effect of Co^{++} Concentration on the Enzymatic Hydrolysis.

A solution containing 0.1M acetyl-DL-methionine, Taka-diastase extract and various concentrations of cobalt chloride was incubated at 37° and pH 6.8, for 21 hours.

2) Practical Application It is evident from the above experiments that Co^{++} activates the enzymatic hydrolysis. Although, in the practical resolution, it is desirable to employ a higher substrate concentration and a less amount of enzyme preparation. So, in order to confirm whether or not the activated enzyme with the addition of Co^{++} advantageously accomplishes, without denaturation, the asymmetric hydrolysis of the susceptible component of the substrate than that of without activation, practical resolution process was carried out with and without the addition of Co^{++}. The reaction mixture consisting of 0.6M acetyl-DL-methionine, extract of Taka-diastase (corresponding to 1/20 and 1/40-weight of the substrate) and 6×10^{-4}M Co^{++} was adjusted to pH 6.8 and incubated at 37°. The rate of hydrolysis was compared with the control run without the addition of Co^{++}.

The results, summarized in Fig. 3, show the advantage of the addition of Co^{++}. The practical

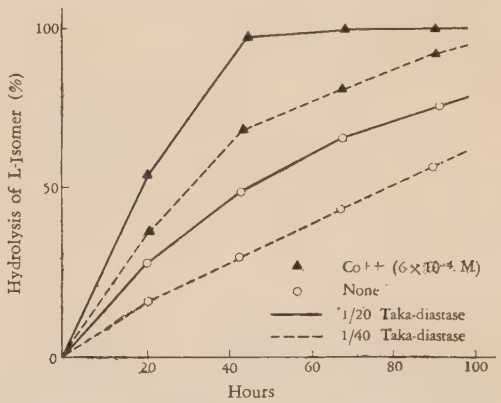


FIG. 3. Asymmetric Hydrolysis of Acetyl-DL-methionine in the Presence and Absence of Co^{++}.

Solutions containing 0.6M neutralized acetyl-DL-methionine and an extract of Taka-diastase (1/20 and 1/40-weight of the substrate) with and without the addition of Co^{++} were incubated at 37°, and pH 6.8.

isolation of L-methionine was also carried out by the method as described in the previous paper. The L-methionine, obtained in the same yield as by the previous method, was optically pure and showed no contamination with Co^{++}, $[\alpha]_D^{25} = +23.0°$ (C 3, N·HCl). *Anal.* Found: C, 40.01; H, 7.23; N, 9.48. Calcd. for $C_5H_{11}NO_2S$: C, 40.25; H, 7.43; N, 9.39. So the enhancement of enzymatic hydrolysis by Co^{++} can be said to be a very useful procedure for the shortening of incubation-time and the curtailment of enzyme preparation in the resolution of acetyl-DL-methionine for preparative purpose.

"Comparison of Renal and Mold Acylases in Metal Activation"

Greenstein and his coworkers have made extensive studies on the hog kidney enzyme which hydrolyzes acyl amino acids and named the purified enzyme acylase 1. They investigated the effect of Co^{++} towards the hyrolysis of chloroacetyl and glycyl-amino acids with this enzyme[2], and reported that the hydrolytic rate of chloroacetyl-L-methionine was inhibited by all concentrations of Co^{++} applied namely, from 10^{-2} to 10^{-4} M (percent decrease −62 to −15). The authors recognized that similary with renal acylase, the enzyme produced by mold is capable of hydrolyzing acyl amino acid, although the hydrolysis of acetyl-DL-methionine by the enzyme was markedly accelerated by the presence of Co^{++}.

In order to ascertain the question whether this discrepancy between renal and mold acylases with regard to the effect of Co^{++}, is due to the difference of the characters of both enzymes or either due to the difference of the substrates, the authors have investigated the hydrolysis of acetyl-DL-methionine and chloroacetyl-DL-methionine by employing hog kidney acylase and mold acylase under the same conditions which were employed by Greenstein.

As for mold acylase, the partially purified preparation described in the previous chapter and an extract of wheat bran cultivated with *Aspergillus oryzae* No 9[3] were employed; Renal acylase 1., was prepared from hog kidney[4]. The enzyme solution and Veronal buffer were preincubated with cobalt chloride (final concentrations, 10^{-3} and 10^{-4} M) at 37°, for 5 minutes before adding the substrate.

The neutralized substrate solution, composed of 0.05 M acetyl-DL-methionine and 0.025 M chloroacetyl-L-methionine, was added and it was incubated from 1 to 2 hours. Simultaneously, the enzyme reactions in the absence of Co^{++} were performed for the controls. The data are shown as percent increase or decrease in their rates and are summarized in Table IV.

With acylase 1, the hydrolysis of chloroacetyl-L-methionine was apparently inhibited by Co^{++}, which coincides with the report of Greenstein; although, in the case of acetyl-DL-methionine, the inhibitory effect of Co^{++} was found to be less than that of the former substrate. While, in the case of mold acylase, the hydrolytic rates of the both substrates were markedly accelerated by either partially purified preparation or crude extract in the presence of Co^{++}. It is of interest to note that, renal and mold acylases, which hydrolyze acyl amino acid, behave differently towards Co^{++} in the hydrolysis of chloroacetyl-L-methionine under the conditions employed. The authors are now study-

TABLE IV
EFFECT OF Co^{++} ON THE RENAL AND MOLD ACYLASES

Substrate	Concentration of Co^{++} (M)	Renal Acylase 1	Mold	
			Extract	Purified prep.
Acetyl-DL-methionine	0	100	100	100
	10^{-3}	90	123	155
	10^{-4}	97	122	145
Chloroacetyl-DL-methionine	0	100(332*)	100(173*)	100(215*)
	10^{-3}	70	126	141
	10^{-4}	75	115	120

After 1ml of enzyme solution, 0.7ml of Veronal buffer and 0.3ml of cobalt chloride (final concentrations, 10^{-3} and 10^{-4}M) were preincubated at 37° for 5 minutes, 1ml of the neutralized substrate (0.05M acetyl-DL-methionine and 0.025M chloroacetyl-L-methionine) was added and incubated at 37° and pH 7, for 2 hours.
* Values are expressed as par cent of acetyl-DL-methionine.

2) K.R. Rao, S.M. Birnbaum, R.B. Kingsley, and J.P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

3) Preparation of the extract will by described in the successive report.

4) S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J.P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

ing the enzyme properties of mold acylase, especially of substrate specificity aiming to compare them with other acyl amino acid hydrolyzing enzymes such as renal acylase and carboxypeptidase. These results will be published in the near future.

Acknowledgements. The authors are grateful to prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa. of this Laboratory for their helpful advice and encouragement throughout the course of this work. Thanks are also due to Messrs. T. Ishikawa and M. Kisumi for their assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 296~299, 1957]

Studies on Amino Acids. VI.

Studies on the Enzymatic Resolution (V). Enzymatic Resolution of DL-Lysine (2)*

By Ichiro CHIBATA, Akira WATANABE and Shun'ichi YAMADA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

Received April 10, 1957

The advantageous resolution procedure of DL-lysine previously reported, consisting of the enzymatic hydrolysis of ε-benzoyl-α-acetyl-DL-lysine with mold enzyme preparation was innovated. Susceptibility of ε-benzoyl-α-acetyl-DL-lysine towards various enzyme preparations such as kidney, pancreas extracts, renal acylase and carboxy-peptidase, was investigated. As a result, it was found that although the substrate was readily hydrolyzed by the mold enzyme preparations, the other enzyme preparations showed, almost no, if any, hydrolytic activity.

Since there had not been any procedure completely satisfactory for the optical resolution of DL-lysine, the authors studied the enzymatic resolution of DL-lysine, such as the asymmetric synthesis of acyl L-lysine anilide and asymmetric hydrolysis of acyl DL-lysines. As a result, the procedure consisting of the enzymatic hydrolysis of ε-benzoyl-α-acetyl-DL-lysine was found to be an advantageous method for the resolution of DL-lysine.

Namely, the advantages of this procedure is as follows: a) The substrate can be easily prepared from the intermediate of the synthesis of DL-lysine; b) the substrate can be readily asymmetrically hydrolyzed by mold enzyme; c) the separation of the hydrolyzed antipode is very simple, for benzoyl-L-lysine crystallizes out during the incubation procedure. These results were reported in our previous paper[1].

The present paper delivers the results obtained by the further study of this enzymatic hydrolysis of the acyl DL-lysine such as metal activation and susceptibility of the substrate to several enzyme preparations.

*Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Kyoto, April 21, 1956.

**Honjo-kawasaki-cho, Oyodo-ku, Osaka.

1) I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20**, 174 (1956).

EXPERIMENT AND RESULTS

Effect of pH

The effect of the pH to the asymmetric hydrolysis of ε-benzoyl-α-acetyl-DL-lysine by the enzyme preparation produced by *Aspergillus oryzae* was investigated. Namely, to the neutralized substrate solution, Taka-diastase extract and phosphate buffer of various pH were added and incubated. The liberated amino-N was determined by the ninhydrin colorimetric method

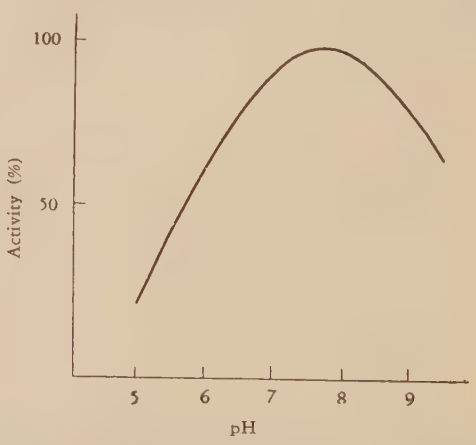


FIG. 1. Effect of pH on the Enzymatic Hydrolysis of ε-Benzoyl-α-acetyl-DL-lysine.

A mixture of 2ml of 0.025M neutralized substrate solution, 2ml of M/15 phosphate buffer and 1ml of Taka-diastase extract (corresponding to one-tenth weight of the substrate) was incubated at 37°, for one hour.

and the result was expressed as percent activity of the control at the optimal pH (Fig. 1). As shown in the Figure, the optimal pH for the enzymatic hydrolysis was found to be in the range 7.0 to 7.5.

Effect of Metal Ions

In a previous paper[2] the effect of various metal ions to the hydrolysis of acetyl-DL-methionine by the enzyme produced by *Aspergillus oryzae* was studied. Since it is possible that the influence of metal ions varies with difference of the substrates, so the experiments employing ε-benzoyl-α-acetyl-DL-lysine as the substrate were carried out. Although both shorter incubation by the addition of 10^{-4} M metal chlorides and prolonged incubation in the presence of 10^{-3} M concentration were performed, no different influence was observed. As listed in Table I, Mn^{++} showed an inhibitory effect while Co^{++} apparently enhanced the enzymatic hydrolysis. The inhibitory effect of Zn^{++} observed in the case of acetyl-DL-methionine was not evident under the conditions employed in this experiment. Presence of Co^{++} activated the enzyme action from 1.8 to 2 times that of the controls, in both substrates.

Table I
Effect of Metal Ions on the Enzymatic Hydrolysis of ε-Benzoyl-α-Acetyl-DL-Lysine

	Activity in the Presence of the Ions							
Exp. No.	None	Mg	Ca	Zn	Sr	Mn	Co	Ni
1*	100	105	104	98	100	60	185	86
2**	100	107	102	102	104	22	178	91

*A solution containing 0.01M neutralized substrate, Taka-diastase extract and 10^{-4} metal chloride was incubated at 37° and pH 7.5, for 1 hour.

**A solution containing 0.1 M neutralized substrate, Veronal buffer, Taka-diastase extract and 10^{-3} metal chloride was incubated at 37° and pH 7.5, for 24 hours.

Effect of Cobalt Ion Concentration

Since the results of the above experiments indicate that Co^{++} accelerates the enzyme reaction, the effect of various concentrations on the enzymatic hydrolysis by a dialized extract of Taka-diastase was investigated. The results, summarized in Fig. 2, disclosed that the optimal concentration for the activation was in the range of 10^{-4} M to 10^{-3} M, and concentration higher than 10^{-2} M retarded the reaction. This observation coincides with the results observed in the enzymatic hydrolysis of acetyl-DL-methionine with a Taka-diastase extract.

2) I. Chibata, A. Watanabe and S. Yamada, This Bulletin, **21**, 291 (1957).

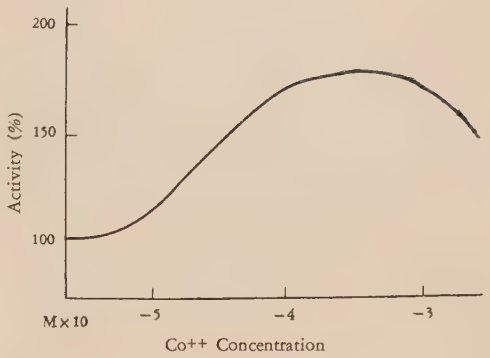


FIG. 2. Effect of Co^{++} Concentration on the Enzymatic Hydrolysis.

A solution containing 0.01M ε-benzoyl-α-acetyl-DL-lysine, dialyzed extract of Taka-diastase and various concentrations of cobalt chloride was incubated at 37° and pH 7.0, for 2 hours.

Practical Resolution Procedure

It is always desirable from the economical standpoint to obtain a maximum yield of product in a minimum incubation time for the practical resolution of racemic amino acids. So, the previously reported procedure of enzymatic hydrolysis of ε-benzoyl-α-acetyl-DL-lysine was modified and carried out in the presence of Co^{++}. To a neutralized solution of the substrate (final concentration, 0.2 M), an enzyme solution (corresponding to one-fortieth weight of Taka-diastase to the substrate) and cobalt chloride (final concentration, 10^{-3} M) were added. The digestion mixture was adjusted to pH 7.0, if necessary, and incubated at 37°. A simultaneous run for the determination of the progress of the reaction indicates that the reaction completed within forty hours. The crystals of ε-benzoyl-L-lysine separated during the incubation were collected by filtration; yield was over 90%. The chemical hydrolysis with hydrochloric acid and subsequent treatment with ion exchange resin as described in the previous paper[1] gave L-lysine monohydrochloride; yield around 85%, $[\alpha]_D^{25} = +20.6$ (C. 3,5 N-HCl). *Anal.* Found: C, 39.00; H, 8.62; N, 15.12. Calcd. for $C_6H_{15}N_2O_2Cl$: C, 39.46; H, 8.28; N, 15.34.

The analysis of the product showed high optical purity and no contamination with Co^{++}. As in the case of the enzymatic resolution of acetyl-DL-methionine, the addition of Co^{++} is an advantageous innovation of the preparative purpose.

Susceptibility to the Pancreatic and Renal Enzymes

Apart from the authors experiments with mold

enzyme, the attempt by Utzino and Yoneya[3] with hog kidney extract has been the only known report on the enzymatic hydrolysis of the ε-benzoyl-α-acetyl-DL-lysine. According to their report, the substrate was completely resistant to the hog kidney enzyme. Therefore the authors reinvestigated their experiments and also performed the comparative study of the hydrolytic activity of the pancreatic, renal and mold enzyme preparations to the substrate.

i) Hydrolysis by Hog Kidney Extract At first, following the report by Utzino and Yoneya, the hydrolysis by hog kidney extract was determined by the formol titration.

Fresh frozen hog kidney was ground with acetone, filtered and dried. The powder was then stirred for 30 minutes with 7 times its weight of water, and filtered. The filtrate was used as the hog kidney extract. The digestion mixture of the neutralized substrate solution and the extract was incubated and the reaction mixture was titrated at proper intervals. A simultaneous run with Taka-diastase extract was performed for the sake of comparison.

The results are shown in Table II, which indicates that the hydrolysis did not proceed by hog kidney extract as reported, although the coloring of the digestion mixture made the end point of titration indistinct.

TABLE II
ENZYMATIC HYDROLYSIS OF ε-BENZOYL-α-ACETYL-DL-LYSINE

Enzyme	Incubation time		
	20 hrs.	68 hrs.	116 hrs.
Kidney ext.	5.6%+	5.6%	4.2%
Taka-diastase ext.	26.5%	44.0%	72.0%

A mixture of 5ml of 0.1M neutralized substrate solution, 10ml of water and 10ml. of enzyme solution was incubated at pH 7 and 37°. After appropriate incubation, 4ml of the digestion solution was taken and determined by formol titration with N/10 sodium hydroxide.
+Percentage of hydrolysis of the susceptible antipode.

ii) Hydrolysis by Renal, Pancreas and Mold Extracts To obtain further accurate results, the comparative studies were performed by the ninhydrin method.

As animal source enzymes, the above mentioned hog kidney extract prepared from fresh frozen beef pancreas followed by the former preparation were employed. Besides the extract of Taka-diastase, the extracts of *Aspergillus oryzae* No. 9 and *Penicillium vinceceum* cultivated on wheat bran were also tested as mold enzyme preparations. (The detail of the preparation of the extracts will be described in the next report of this serial work).

3) S. Utzino and T. Yoneya, *Ber.*, **85**, 860 (1952).

TABLE III
ENZYMATIC HYDROLYSIS OF ε-BENZOYL-α-ACETYL-DL-LYSINE

Enzyme prep.	Incubation time		
	20 hrs.	48 hrs.	96 hrs.
Kidney ext.*	—	—	0.5%
Pancreas ext.*	—	—	0.6%
Taka-diastase+	38%	59%	81 %
Oryzae. No. 9.+	46%	64%	80 %
Pen. vinaceum	41%	63%	78 %

*A mixture of 4ml of 0.1M neutralized substrate solution, 2ml of M/15 phosphate buffer, 1.5ml of water and 0.5ml of enzyme solution was incubated at pH 7, and 37°.
+A mixture of 5ml of 0.02M neutralized substrate solution, 2.5 ml of M/15 phosphate buffer and 25ml of enzyme solution was incubated at pH 7.5, and 37°.

The results, summarized in Table III, show that the substrate is neither hydrolyzed by hog kidney extract nor by beef pancreas extract, although the substrate is readily hydrolyzed by all of the mold preparations employed.

iii) Susceptibility to Renal Acylase and Carboxypeptidase From the above experiments, it was disclosed that the ε-benzoyl-α-acetyl-DL-lysine was not hydrolyzed by kidney and pancreas extracts. So the behavior of the substrate towards further purified enzymes such as carboxypeptidase and renal acylase, which were known to show hydrolytic activity toward the acyl amino acids, were investigated.

The renal acylase was prepared from fresh hog kidney according to the method described by Greenstein and his co-workers[4]. Carboxypeptidase was prepared from beef pancreas by the method of Anson[5].

TABLE IV
ENZYMATIC HYDROLYSIS OF ε-BENZOYL-α-ACETYL-DL-LYSINE

Enzyme	Incubation time		
	20 hrs.	44 hrs.	72 hrs.
Acylase 1	2.0%	3.7%	7.0%
Carboxypeptidase	0 %	0 %	0 %
Taka-diastase	30.0%	62.0%	85.0%

A mixture of 4ml of 0.02M neutralized substrate solution, 2 ml of M/15 phosphate buffer and 2ml of enzyme solution was incubated at pH 7.5 (pH 7.0 for acylase 1).

4) S.M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).
5) M.L. Anson, *J. Gen. Chem.*, **20**, 663, 777 (1937).

The substrate was incubated with these enzyme preparations and the Taka-diastase extract as the control. The extent of hydrolysis was determined by the ninhydrin method. The results listed in Table IV, indicate that the substrate was completely insusceptible to carboxypeptidase and it seemed slightly susceptible to acylase I under those conditions employed by the authors. However, the hydrolysis by renal acylase was significantly lesser than that of the mold enzyme preparation.

It is of interest to note that three enzymes, i. e., carboxypeptidase, renal and mold acylases, which were known to act on acyl amino acids, were differentiated by their behavior towards ε-benzoyl-α-acetyl-DL-lysine.

Acknowledgements. The authors are grateful to prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa of this Laboratory for their helpful advice and encouragement throughout the course of this work. Thanks are also due to Messrs. T. Ishikawa and M. Kisumi for their assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 300~303, 1957]

Studies on Amino Acids. VII.

Studies on the Enzymatic Resolution (VI). A Survey of the Acylase in Molds*

By Ichiro CHIBATA, Tsutomu ISHIKAWA and Shun'ichi YAMADA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

Received April 10, 1957

The distribution of activity hydrolyzing acyl amino acids was investigated, so as to obtain an advantageous enzyme source for the resolution of amino acids. For this purpose, a number of molds such as *Aspergillus, Penicillium, Rhizopus, Mucor, Abshidia,* and *Cunninghamella* were cultivated on wheat bran and the extracts were tested in relation to their hydrolytic activity of acyl amino acids. Although almost all molds yielded acylase more or less, high activity was shown in cases of *Aspergillus* and *Penicillium.* As one of the most suitable strains, *Aspergillus oryzae* No. 9 was chosen and the optimum cultural conditions for acylase formation was also inveitigated.

On the asymmetric hydrolysis of acyl derivatives of amino acids, Greenstein and his co-workers have made extensive studies with renal acylase and have succeeded in the optical resolution of a number of racemic amino acids. Neuberg and Mandl[1] have reported on the asymmetric hydrolysis of acyl amino acids employing several commerical enzyme preparations including Taka-diastase. Michi and Nonaka[2], have also studied on the acylase activity in molds.

The authors[3] previously reported the successful resolution of methionine and lysine with mold enzyme preparation. To achieve advantageous utilization of mold as an enzyme source, a number of molds, especially, *Aspergillus oryzae*, were investigated to be employed for the resolution of racemic acyl amino acids. As a result, the most suitable mold was chosen and various factors influencing the production of the acylase activity were investigated to ascertain the optimum cultural conditions.

MATERIALS AND METHODS

Compound Used Acetyl-DL-methionine was synthesized from DL-methionine, by the method previously reported.

Organisms Organisms used were chosen from *Aspergillus, Rhizopus, Mucor, Abshidia,* and *Cunninghamella.* These molds were supplied through the courtesy of Dr. S. Fukui, Institute of Applied Microbiology, University of Tokyo.

Cultivation A mixture of 6 g of wheat-bran and 4.2 ml of tap water in a 200 ml flask was sterilized by autoclaving, then, a loop of the stock-culture of the tested strain was innoculated and it was cultivated at 27°. After an appropriate period of incubation, the mixture was shaked for 1 hour with 100 ml of distilled water and filtered with the aid of Celite. The extracts were used as the enzyme solution. Results of the cultivation on wheat bran were somewhat variable due to the minor difference of conditions. So, the experiments were usually repeated several times, and average values are described below.

Assay of Enzyme Activity As the acyl derivatives of amino acids were hydrolyzed by acylase, the liberated amino nitrogen was determined by the Van Slyke method. In these experiments, acetyl-DL-

*Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Kyoto, September 22, 1956.

**Honjo-Kawashi-cho, Oyodo-ku, Osaka.

1) C. Neuberg and I. Mandl, *Enzymologia*, **14**, 128 (1950); U.S. pat. 2, 511, 867 June 20 (1950).

2) K. Michi and H. Nonaka; *J. Agr. Chem. Soc. Japan*, **28**, 343 (1956).

3) S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Japan*, **75**, 113 (1955). I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20**, 174 (1956).

methionine was employed as the substrate. Namely, a mixture of 2.5 ml of 0.4 M neutralized substrate solution, 1 ml of Sörensen phosphate buffer (pH 6.8) and 1.5 ml of enzyme solution was filled up to 10 ml, and subsequently adjusted to pH 6.8, if necessary. The digestion solution was incubated at 37° for 24 hours and the increased amino-nitrogen was determined. The activity was compared with increased mg of amino nitrogen or either the extent of hydrolysis.

RESULTS AND DISCUSSION

Comparison of the Hydrolytic Activity Produced by Molds According to the assay described above, hydrolytic activity produced by various mold strains were compared. Cultivation was carried out for 6 days except in the case of *Aspergillus*, which was cultivated for 4 days. As shown in the Table I, almost all the molds tested, exhibited more or less acylase activery. The strains which showed higher activity (for convenience, distinguished by the asterisk in the Table) were mainly found in the strains of *Aspergillus oryzae* and *Penicillium*. Generally speaking, cultivation of *Aspergillus oryzae* is

Table I
Survey of Acylase Activity Produced by Molds

Strain		Activity+ mg	Strain		Activity mg
Asp. oryzae No.	1	0.20	*Asp. oryzae* No.	117*	0.40
〃	5	0.32	〃	147	0.25
〃	7	0.33	〃	161	0.37
〃	8	0.37	〃	I	0.08
〃	9*	0.52	〃	II*	0.43
〃	10	0.25	*Asp. tamari*	3	0.27
〃	12*	0.44	〃	19	0.37
〃	21	0.38	*Asp. fumigatus*	1	0.35
〃	22	0.26	〃	2	0.17
〃	32	0.29	*Asp. niger*		0.24
〃	33	0.32	*Asp. wentii*		0.25
〃	36*	0.42	*Asp. terreus*		0.27
〃	37	0.27	*Asp. parasites*		0.30
〃	38	0.29	*Asp. nidulaus* Eidan		0.33
〃	39	0.29	*Asp. clavatus* Deanaziens		0.32
〃	46	0.38	*Asp. rugulosus* 1335		0.08
〃	51	0.34	*Asp. sydowi*		0.01
〃	52*	0.44	*Asp. flavus*		0.23
〃	53	0.17	*Asp. ochraceus* Wilhelm		0.27
〃	66*	0.46	*Asp. melleus**		0.44
〃	67	0.27	*Pen. corymbiferum**		0.45
〃	68	0.24	*Pen. vinaceum**		0.49
〃	69*	0.40	*Pen. citrinum*		0.13
〃	71*	0.43	*Pen. notatum*		0.17
〃	73	0.24	*Rhizopus javanicus*		0.13
〃	75*	0.48	*Rhizopus peka*		0.23
〃	76*	0.43	*Mucor circinelloides*		0.36
〃	78	0.30	*Mucor jansseni*		0.15
〃	83	0.31	*Absidia orchidis*		0.07
〃	87	0.30	*Absidia sptata*		0.03
〃	100	0.27	*Cunninghamella blakesleana*		0.08
〃	107*	0.40	*Cunninghamella echinulata*		0.14

*activity is expressed as the increased mg of amino-nitrogen per ml of digestion solution, after 24-hours' incubation.

easily performed, so one of the strains possesing highest activity, *Aspergillus oryzae* No. 9, was chosen as the advantageous mold for the enzyme source, and the various conditions of cultivation were investigated with this strain for practical purposes.

On the Influence of Cultural Conditions

i) Effect of Water When cultivated in flask, the addition of 100% water to wheat bran resulted in partial clotting and adherance to the glass-wall and disturbance of uniform growth of the mold. The addition of 70% of water to the wheat bran gave better results than that of adding 50% of water, although this should be particularly considered, when cultivated in a petri-dish or "Koji-buta".

ii) Effect of Rice-hulls It was expected that the addition of rice-hulls to the wheat bran would be effective to promote the formation of hydrolytic activity during cultivation.

So, the various amounts of rice hulls were added and cultivated under the conditions previously described. Then, the hydrolytic activity of the extracts was compared.

As shown in Table II, after cultivation for 5 days the addition was found to be very effective and formation of hydrolytic activity was increased by one and one-half times, although the extracts became slightly turbid and colored. It was possible, however, that as the cultivation without addition was delayed more than that with the addition, and the formaction of acylase activity could not reach its maximum level. So prolonged cultivation, 10 days, was carried out. Since the result was the same as that in the case of the shorter period, the addition of rice hulls to wheat bran proved to be an effective procedure for practical purposes.

TABLE II
EFFECT OF ADDITION OF RICE-HULLS ON THE ACYLASE FORMATION

No.	Culture media			Period of Cultivation	
	Wheat Bran	Rice-hulls	Water*	5 days	10 days
	g	g	ml		
1	6	0	4.2	100+	123
2	6	1.2	5.0	112	
3	6	3.0	6.3	137	
4	6	6.0	8.4	149	185

*70% of water is added to the solid.
+Relative activity of the extract.

iii) Effect of Nitrogen Sources In order to find out the influence of nitrogen sources, experiments were carried out on the addition of peptone and extract of soy bean meal.

a) Peptone: Various amounts of 10% solution of peptone were added to the wheat bran and cultivation was carried out for 8 days; The results obtained are shown in Table III.

b) Extract of Soy Bean Meal: The soy bean meal was refluxed with 0.2% sodium hydroxide solution and filtered. The filtrate was neutralized with hydrochloric acid and was used as a 10% extract. The results are shown in Table IV.

TABLE III
EFFECT OF ADDITION OF PEPTONE ON THE ACYLASE FORMATION

No.	Culture Media			Relative Activity
	Wheat	Peptone	Water	
	g	ml	ml	
1	6	0	4.2	100
2	6	0.3	3.9	102
3	6	0.6	3.6	100
4	6	1.2	3.0	105
5	6	1.8	2.4	104
6	6	3.0	1.2	107

TABLE IV
EFFECT OF ADDITION OF EXTRACT OF SOY BEAN MEAL ON THE ACYLASE FORMATION

No.	Culture Media			Activity
	Wheat Bran	Extract	Water	Activity
	g	ml	ml	
1	6	0	4.2	100
2	6	0.3	3.9	95
3	6	0.6	3.6	98
4	6	1.2	3.0	102
5	6	1.8	2.4	101
6	6	3.0	1.2	99
7	6	4.2	0	104

Throughout these experiments concerning the addition of nitrogen compounds, no significant influence on the formation of acylase activity was observed.

iv) Effect of Period of Cultivation On the cultivation of *Aspergillus orzyae* No. 9 on wheat bran, the relation of the period of cultivation and formation of acylase activity was studied. Considering the use of them as enzyme sources, conditions of preservation were also investigated.

Namely, under the standard conditions, the formation of activity was assayed along with the progress of cultivation. As shown in Fig 1, formation of activity reached its maximum level in 6 days. Further, prolonged incubation resulted in no significant change of activity.

Cultured brans at 27°, for 10 days were prepared. After preservation for 50 days at 27°, room temperature (15–25°), and placed in a refrigerator (1–5°), the samples showed 90.0%, 92.6% and 98.1%, respectively, of the initial activity. Therefore the preservation of the cultivated brans may be carried out without any difficulty.

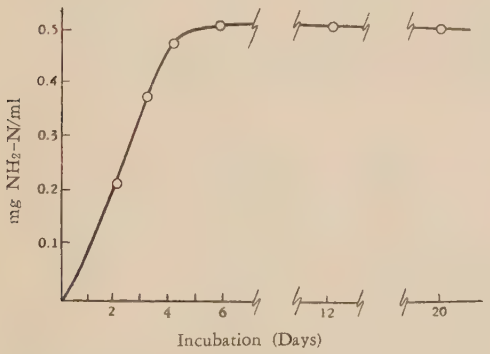


FIG. 1. Formation of Acylase Activity on the Cultivation of Wheat Bran.

On the Extraction of Acylase In the solid culture on wheat bran, the procedure of extraction was examined so as to utilize the activity as much as possible. For this purpose, several extraction procedures of cultured mixture prepared by the standard cultivation were carried out, as follows:

a) shaking with water for 1 hour, as described in the previous chapter;

b) after grinding with a blendor, shaken for 1 hour with water;

c) after freezing with dry ice, thawed and shaken for 1 hour with water;

d) after grinding with a blendor, 20% *n*-butanol was added under stirring at 0∼–5° and shaken further for 1 hour, then the aqueous layer was used as extract. Comparison of the effectiveness of extraction, was shown by the relative activity of the extracts, in which the activity of method (a) was taken as 100. The results summarized in Table V, show that method (b), that is, grinding with a blendor and extraction by shaking with water for 1 hour, proved to be a satisfactory procedure.

TABLE V
EFFECT OF VARIOUS EXTRACTION PROCEDURES OF MOLD ACYLASE

Exp.	Extraction Procedure	Effectiveness* (%)
a)	Shaking with water	100
b)	Grinding and shaking with water	127
c)	Freezing, thawing, and shaking with water	110
d)	Grinding and shaking with aqueous n-BuOH	122

*Relative activity of the extract.

Acknowledgements. The authors are grateful to Prof. H. Mitsuda of Kyoto University, and Dr. M. Fujisawa of this Laboratory for their helpful advice and encouragement. They also wish to thank Dr. S. Fukui of the Institute of Applied Microbiology, University of Tokyo, for his generous supply of the microorganisms, used in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 304~307, 1957]

Studies on Amino Acids. VIII.

Studies on the Enzymatic Resolution (VII). Specificity of Mold Acylase*

By Ichiro CHIBATA, Tsutomu ISHIKAWA and Shun'ichi YAMADA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

Received April 10, 1957

With the mold acylase preparation, optimal pH and relative activity to the various acyl amino acids were investigated. As a result, a difference of optimal pH was observed between acetyl and chloroacetyl derivatives. A comparison of the susceptibility disclosed that mold preparation has either a wider or loose specificity than renal acylase. Namely, besides aliphatic amino acids, the acyl derivatives of basic amino acids and aromatic amino acids were readily hydrolyzed by the mold preparation. These results indicate that the mold acylase preparation can be used for the resolution of a great variety of amino acids. As an example of this, the resolution of acetyl-DL-tryptophan was presented.

In the previous paper[1], the authors have reported on the survey of acylase activity in molds and several strains of *Aspergillus oryzae* and *Penicillium* were found to show high hydrolytic activity to acyl amino acid. In this paper, with the mold acylase preparation from *Aspergillus oryzae* No. 9 and *Pencillium vinaceum*, the optimal pH and relative activity towards various acyl amino acids were investigated to acquire fundamental knowledge concerning the enzymatic resolution of amino acids. Comparison with other hydrolytic enzymes of acyl amino acids such as renal acylase and carboxypeptidase revealed several interesting results.

MATERIALS AND METHODS

Substrates The N-acetyl derivatives employed in this work were prepared by the acetylation of respective amino acid with acetic anhydride in acetic acid or in an alkaline solution except acetyl-DL-tryptophan, which was prepared by partial hydrolysis and decarboxylation of ethyl α-acetamino-α-carbethoxy-β-(3-indole)-propionate, an intermediate of the synthesis of DL-tryptophan.

Preparation of N-chloroacetyl derivatives was carried out by the interaction of chloroacetylchloride and amino acids in alkaline medium.

Mold Acylase Preparations For the purpose to carry out the enzyme assay, the mold acylase was prepared as follows: As under the conditions described in the previous paper[1], 340 g of wheat bran innoculated with *Aspergillus oryzae* No. 9 was incubated for 8 days, at 27°. The cultured mixture was extracted by shaking with water for 1 hour, and was filtered with the aid of Celite. The filtrate was brought up to 0.6 saturation with ammonium sulfate at pH 6.0 and then centrifuged. The precipitate was dissolved in water and dialyzed against flowing cold water for 3 days. The dialyzed solution was treated with a 0.3-volume of chilled acetone at −2°. The precipitate was separated by centrifugation and discarded. The supernatant solution was chilled again, and treated with additional 0.6 volume of acetone (based on the original volume). The precipitate was separated by centrifugation and dried. The dried preparation weighed 950 mg. Activity of the preparation, by the assay using acetyl-DL-methionine as the substrate, was 15.75 μM per hour, per mg of the preparation; the yield of total activity was 63%.

Preparation of *Penicillium vinaceum* was also carried

*Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Kyoto, September 22, 1956.

**Honjo-Kawashi-cho, Oyodo-ku, Osaka.

1) I. Chibata, T. Ishikawa, and S. Yamada: This Bulletin, **21**, 300 (1957).

out in the same manner, as in the previous case. From 370 g of innoculated wheat bran, 1,300 mg of dry preparation was obtained whose activity was 10.31 μM per hour per mg of the preparation; the yield of total activity from the filtrate was 48%.

Enzyme Reaction The enzyme reaction was carried out substantially, following the methods employed by Greenstein and his co-workers. Namely, digests composed of 1 ml of enzyme solution, 1 ml of phosphate buffer (of appropriate pH), and 1 ml of neutralized 0.05 M racemic substrate (0.025 M for the L-form) were incubated at 37° for 2 hours. Liberated amino acids were measured by the ninhydrin method. The rate of hydrolysis was determined from the splitting of the susceptible substrate which was between 10 to 30 per cent.

RESULTS AND DISCUSSION

Influence of pH According to the report[2] on the renal acylase, the optimum pH for the hydrolysis of acetyl methionine and chloroacetyl alanine was about 7.2; for the hydrolysis of acylated derivatives of glutamic acid it was abuot 6.5. The effect of pH on the hydrolytic rate of various acyl amino acids by the mold preparation was studied and the optimal pH was determined.

The results are summarized in Table I. It is of interest to note that, except in the case of acyl glutamic acids, the optimum pH for the hydrolysis of acetyl derivatives and chloroacetyl derivatives somewhat differed. Namely, the acetyl derivatives were generally hydrolyzed at a maximal velocity over the range of pH 6.5 to 7.5, mainly around pH 7.0, although, the optimum for the chloroacetyl derivatives shifted to the acidic side, that is, pH 5.0~6.0. No significant difference of the optimal pH was observed between *Aspergillus oryzae* and *Penicillium* preparations, except that of the alkaline-side optimum pH of the *Aspergillus oryzae* preparation for acetyltryptophan.

Table I
Optimal pH of Mold Acylase for the Hydrolysis of Acyl Amino Acids

Acyl derivs.	Acetyl		Chloroacetyl	
	Asp.	Pen.	Asp.	Pen.
Alanine	6.0	6.5	5.5	5.5
Leucine	6.5	7.0	6.0	6.0
Methionine	7.0	6.5	6.0	6.0
Glutamic acid	5.0	5.5	5.0	5.5
Asparfic Acid	5.5	5.0	5.0	5.0
ε-Benzoyllysine	7.0	7.0	5.0	5.0
Tryptophan	8.0	6.5	5.0	5.0
Phenylalanine	7.5	7.0	5.5	6.0
Tyrosine			6.0	5.5

2) S.M. Birnbaum, L. Levintow, E.B. Kingsley and J.P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

Substrate Specificity of Mold Acylase Susceptibility of the various acyl amino acids towards the mold preparation was studied in their initial rate of hydrolysis under standard conditions. The results obtained are summarized in Table II, the data of the renal acylase are also listed for comparison. For the convenience of interpreting the results, the initial

Table II
Relative Susceptibility of Acyl Amino Acids to Acylases

Acyl Amino Acid	Hog Kidney[2,5]		Mold	
	Homogenate	Acylase I	*Asp. oryz.*	*Pen. vin.*
Ac-Alanine	21	13	73	56
Clac- 〃	72	48	144	89
Ac-Leucine	27	22	31	21
Clac- 〃	103	68	46	29
Ac-methionine	100	100	100	100
Clac- 〃	422	410	180	171
Glycyl 〃	517	62	26	10
Ac-Glutamic Acid	21	13	37	27
Clac- 〃	78	53	53	44
Ac-Aspartic Acid	1.8	<0.1	10	18
Clac- 〃	5	<0.1	10	12
diAc-Lysine		0.3		
diClac- 〃	1.6	0.6		
ε-Bz-α-Ac- 〃			83	18
ε-Bz-α-Clac- 〃	1	0.1	158	23
Ac-Tryptophan	<0.1	<0.1	74	35
Clac- 〃 〃	0.1	<0.1	63	19
Ac-Phenylalanine	0.3	0.6	145	81
Clac- 〃 〃	4	1.9	215	134
Clac-Tyrosine	1.6	1.4	226	93

Ac: acetyl, Clac: chloracetyl, Bz: benzoyl

rate of hydrolysis was converted to a relative susceptibiltiy, in which the rate of acetyl methionine was taken as the 100 in the respective enzyme preparations.

When asymmetric hydrolysis of acyl amino acid is proceeded by enzymatic action, it is considered to occur at more than 3 points of interaction between the enzyme and substrate, and the relative rate of the enzyme toward various amino acids is influenced by both the electronic and steric natures of the acyl radical and the amino acid residue.

In regard to the hydrolytic activity of pancreas carboxypeptidase toward acyl amino acids, it has been known that only acyl aromatic amino acids are hydrolyzed by the enzyme, except that of aliphatic amino acids. As for the acyl derivatives, chloroacetyl compounds of phenylalanine and tryptophan were hydrolyzed by carboxypeptidase at a rate about 180-times faster than the corresponding acetyl derivatives[3]. According to Smith[4], the electronic effect of acyl radical is influential, that is, the highly electronegative chloroacetyl radical confers much greater susceptibility on the substrate to enzymatic hydrolysis than the acetyl does. Aromatic amino residue, also, has higher affinity to carboxypeptidase. Hence, this enzyme has been used for the resolution of the chloroacetyl derivatives of racemic phenylalanine, tyrosine, and tryptophan.

As shown in the Table, Greenstein and his co-workers[2~5] have conducted extensive studies on the relative rate of renal acylase toward a large number of acyl amino acids, from which it was revealed that the activity of purified renal acylase I. was about thirty-times more active than the crude homogenate for each substrate, except in the case of acylated aspartic acid. Contrary to the action of carboxypeptidase, although renal acylase is most active to the acylated aliphatic amino acid, whereas, acyl derivatives of aromatic amino acids such as tryptophan, phenylalanine and tyrosine and also of basic amino acids such as lysine were significantly less susceptible to the enzyme. Therefore, the steric effect is more influential than in the case of carboxypeptidase, and the balky residue of aromatic amino acid interferes enzyme-substrate interaction. On the other hand, the electronic influence of the acyl radical appears to a lesser extent, that is, the chloroacetyl derivative is always about four-times more susceptible than the acetyl derivative whereas, the corresponding study on the relative rate of mold preparation, disclosed several interesting facts as those which are described below. The mold preparations show comparatively uniform hydrolytic activity to the various acyl amino acids, namely, not only the acyl derivatives of aliphatic amino acids but of aromatic amino acids and basic amino acids, which are less susceptible to the renal acylase, are also readily hydrolyzed by the acylase in molds. So it may be said that fungal acylases provide both substrate specificities of renal acylase and carboxy peptidase in regard to the amino acid residue. On the influence of acyl radical, as seen in the cases of methionine, glutamic acid, ε-benzoyl-lysine, and phenylalanine, the chloroacetyl derivative is hydrolyzed at a rate from one and one-half to two times faster than the acetyl derivative. However, an exception occurred in the case of tryptophan, in which the expected difference of the rates of hydrolysis between acetyl and chloroacetyl derivatives, was not observed. Generally speaking, the influence of acyl radical to the mold preparations was less than that in the case of renal acylase, especially of carboxypeptidase. In the previous paper[6] refering on the enzymatic resolution of DL-lysine, the authors have reported that ε-benzoyl-α-acetyl-DL-lysine was hydrolyzed faster than the α-chloroacetyl derivative. This contradiction with the present result is due to the fact

3) J.B. Gilbert, V.E. Price and J.P. Greenstein. *J. Biol. Chem.*, **180**, 473 (1949).
4) E.L. Smith, *Proc. N.A.S.*, **35**, 80 (1949).
5) W.S. Fones and M. Lee, *J. Biol. Chem.*, **210**, 227 (1954).
6) I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20** 174 (1956).

that in previous experiments, susceptibility was compared at the same pH for all substrates instead of their respective optimal pH. However, the present result does not lessen the any advantageousness of the previously reported resolution procedure employing ε-benzoyl-α-acetyl-DL-lysine as the substrate, because the difference of the susceptibilities to the mold preparation is comparatively less. Furthermore, employment of the acetyl derivative undoubtedly has advantages over the chloroacetyl derivative in practical resolution because of its readiness of preparation and its convenient racemization procedure.

Among the *Aspergillus oryzae* and *Penicillium* preparations, no significant difference was found in the relative rate.

Besides the acyl amino acids listed in the Table, the hydrolysis of the amides of methionine, leucine and lysine was slso carried out with the mold preparations, although, hydrolytic action was very low. The fact that the mold acylase showed wider hydrolytic activity to the various acyl amino acids indicates that with this single enzyme preparation, the optical resolution of a varieiy of racemic amino acids can be achieved.

Resolution of DL-Tryptophan The acyl derivatives of DL-trypophan are not practically hydrolyzed by renal acylase, so Greenstein and his co-workers have succeeded in the resolution of chloroacetyl-DL-tryptophan by employing carboxypeptidase. However, the employment of acetyl-DL-tryptophan as the substrate for the resolution is advantageous over the chloroacetyl derivative, as the former is obtained as the intermediate of the synthesis of DL-tryptophan. Although Neuberg and Mandl[7] carried out the enzymatic hydrolysis of acetyl-DL-tryptophan using the commercial enzyme preparation "Orthozym", much amount of the enzyme preparation was necessary and prolonged incubation such as 10 days was required to complete the reaction.

It was noted from above that the mold preparation can readily hydrolyze both acetyl- and chloroacetyl-tryptophans. So, the practical resolution using acetyl-DL-tryptophan as the substrate was carried out and successful results were obtained.

Fifty ml of the digestion solution containing 3.08 g of acetyl-DL-tryptophan and 55 mg of the *Aspergillus* preparation was adjusted to pH 8. and incubated at 37°. The analysis of a portion of the solution indicated that asymmetric hydrolysis was completed within 24 hours. The digestion solution was made up to pH 5.0, heated once to coagulate protein and then filtered. The filtrate was concentrated in vacuo and the residue treated with hot alcohol to dissolve acetyl-D-tryptophan. The mixture was allowed to stand overnight in a refrigerator, and the separated crude L-tryptophan was collected by filtration; yield. 1.13 g (88.4%). Recrystallization from aqueous alcohol gave colorless pure L-tryptophan; yield. 0.83 g (65%), m.p. 285°, $[\alpha]_D^{28} = -32.0°$ (0.5% in water).

The alcoholic solution containing acetyl-D-tryptophan was concentrated in vacuo. The residue was acidified and extracted with ethyl acetate. Evaporation of the solvent gave acetyl-D-tryptophan; yield 1.2 g (78%) $[\alpha]_D^{28}$ −28° (1% in methanol).

Although in a previous paper[8], the authous have reported the enzymatic resolution procedure of DL-tryptophan, that is the asymmetric hydrolysis of DL-tryptophan amide with pancreatic enzyme, the present procedure in practical resolution seems to be somewhat advantageous over the previous method.

Acknowledgements. The authors are grateful to prof. H. Mitsuda, Kyoto University and Dr. M. Fujisawa, Director of this Laboratory, for their helpful advice and encouragement.

7) C. Neuberg and I. Mandl, *Enzymologia*, **14**, 128 (1950).

8) I. Chibata, S. Yamada and S. Yamada, This Bulletin, **21** 62 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 308~312, 1957]

Studies on Koji

Part I. A New Analytical Method of Koji

By Kisirō Yamamoto

Noda Institute for Scientific Research

Received May 22, 1957

The "Paste-medium" technique, described here, has made the separate treatment of the mycelial-part and the substrate-part of koji materials, possible in the study of koji. The ground koji materials were mixed with water to be brought into the state of paste. The paste-medium, whose surface was flattened in a petri-dish and covered with a piece of nylon-cloth, was sterilized in the usual way, in an autoclave. After inoculation and growth of the koji mold on the surface, the mycelial-part together with a piece of nylon, was stripped off, and the corresponding substrate-part was digged out of the paste-medium.

This new method enables us to investigate the effects of some cultural conditions on the growth of *Asp. soyae* KS.

In this country the koji, in the form of a solid-culture of the koji mold, has long been used in brewing industries for producing Sake, Shōyu and Miso. As for the properties of the koji, a great many workers have been studying this from various aspects. However, it cannot be clearly described what properties of the koji have the most benevolent influence upon the final-products of brewing (i. e., Sake, Shōyu, etc.), because, the nature of the koji is very complex in its interaction between the mycelium and the koji material, and that the estimation of the final-products of brewing depends upon individual sense of taste, and especially, there is no appropriate method for the analytical study of koji.

As a method for the analytical study of koji, two have been reported up to date; one is the so-called asbest-koji method[1,2], and the other the digestion method[3] by diastase. But the former has some difficulty in dealing with the asbest-koji, and the latter cannot be applied to a protein-rich medium. Furthermore, the properties of koji mold cultivated on an agar plate, which is commonly used, may differ in various points from those cultivated on the koji-material.

It is obvious that the characteristics of koji mold itself, developed during the koji-making period have many significant effects on the final-products of brewing[4]. Accordingly, it would be desirable to study with ease, the growth of koji mold on the koji material as it actually happens, and to deal with the change of both components of mold-mycelium and koji materials, respectively.

In this report, a new technique will be proposed which permits us the study of such purpose. *Asp. soyae* is used in this investigation and some discussions on the method are described here.

MATERIALS AND METHODS

1. Preparation of Paste-Medium: Various sorts of koji materials were put into a crusher and made into powder, so fine as to pass through a sieve of 50 mesh, yielding over 95 per cent of the its original quantity. An appropriate weight (8–10 g) of the

1) A.N. Bindal and Sreenivasaya, *J. Sci. & Ind. Research*, **3**, 386 (1945): *C.A.*, **40**, 5579 (1946).

2) K. Sakaguchi, H. Okazaki and M. Takeuchi, *J. Agr. Chem. Soc. Japan*, **29**, 349 (1955).

3) G. Terui and T. Morimoto, *J. Ferm. Tech.* (*Japan*), **34**, 575 (1956).

4) K. Sakaguchi, *Essays on Brewing Vol .3* (*Japan*), 247 (1943).

powder thus produced, was mixed with a certain volume (8–20 cc) of distilled water to make a uniform paste. The paste was then put into a petri-dish (diam. =85 mm, h=20 mm) and the surface was made flat with a spoon. Then, on the paste surface a few pieces (usually 3–4, per dish) of nylon-cloth, each of 25 mm square were pressed closely, though when a giant colony culture of koji mold is to be made, nylon pieces are not necessary.

Petri dishes each containing the paste media mentioned above were set in a steel cylindrical case (diam. = 11 cm, h. = 27 cm, and 10 steps) and sterilized by steam as usual in a cylindrical steel sterilizer (diam. = 13 cm, h. = 40 cm).

2. Inoculation: *Asp. soyae* Sakaguchi et Yamada[5] was cultured on a steamed wheat bran in a flask, from which the conidien were inoculated on the paste-medium mentioned above with a writing brush, which was kept once in a 70 per cent alcohol solution, was sterilized by steam in a sterilized flask. A single touch of the writing-brush on the pre-culture was enough for inoculation per one petri-dish. The brush was rubbed many times against the paste-medium for uniform seeding of spores on the surface. In case of the observation of the speed of mold growth was necessary, inoculation was made through a platinum needle on the central-point of the paste-medium. Then, the dishes were kept reversed in the usual manner, during the period of incubation. The cultural temperature was generally 30°C.

3. The Separation of the Mycelium from the Paste-Medium: A cork-borer was lightly impressed on the mycelium of the mold grown on the nlyon-cloth, a circular mark thus appearing. Then the nylon-cloth with its mycelium was stripped off the paste-medium, and the paste side of the nylon-cloth was washed with some distilled water with much care being taken so that spores might not fly away. Finally, a sample of the mycelial part was obtained by cutting the stripped nylon-cloth with its mycelium along the above-mentioned circular mark with scissors. On the other hand, a sample of the substrate part was obtained by digging out the paste-medium under the circular mark with a borer, to which the washing just described, was added.

4. Analysis: The rate of growth was observed by the diameter of the giant colony grown on the paste-medium. The number of spores was counted with a haemazitometer. The weight of mycelium was determined by drying the mycelial sample for 4 hours at 95°C, and dried weight of nylon-cloth was subtracted from the gross weight. N-content of the mycelium was determined by Kjeldahl-method.

RESULTS

1. Effect of Cultural Temperature: According to the usual methods of culture, whether Koji-agar or Czapeck-agar was employed, there was no clear distinction in their results in the region 30 to 35°C as the cultural temperature in either Koji-agar or Czapeck-agar. But this method using paste-medium of

TABLE I
EFFECT OF CULTURAL TEMPERATURE ON THE MYCELIAL GROWTH OF *ASP. SOYAE* KS IN THREE KINDS OF CULTURE

Kind of culture	Cultural Temperature	Cultural-time (days) 1	2	3	4
Koji-agar plate	25°C	7 mm	18 mm	49 mm	
	30 〃	21 〃	40 〃	63 〃	
	35 〃	20 〃	41 〃	62 〃	
	40 〃	5 〃	14 〃	24 〃	
Czapeck-agar plate	25 〃	5 〃	12 〃	16 〃	21 mm
	30 〃	11 〃	18 〃	24 〃	32 〃
	35 〃	9 〃	16 〃	22 〃	30 〃
	40 〃	3 〃	6 〃	10 〃	15 〃
Paste-medium	20 〃	2 〃	5 〃	10 〃	17 〃
	25 〃	7 〃	19 〃	30 〃	40 〃
	30 〃	8 〃	23 〃	34 〃	46 〃
	35 〃	13 〃	31 〃	47 〃	62 〃
	40 〃	8 〃	15 〃	22 〃	26 〃

5) K. Sakaguchi and S. Yamada, *J. Agr. Chem. Soc. Japan*, **20**, 65, 141 (1945).

TABLE II

EFFECT OF CULTURAL TEMPERATURE ON THE N-CONTENT OF THE MYCELIUM IN PASTE CULTURE

Cultural-time		36 hrs	48 hrs	72 hrs	96 hrs
Cultural temperature	20°C		6.29%	5.90%	6.00%
	30 〃	5.16%	5.04 〃	5.14 〃	5.17 〃
	35 〃	4.88 〃	4.55 〃	4.68 〃	4.91 〃

wheat bran clearly showed 35°C as the optimal temperature (Table I). This temperature was also optimal for both mycelial weight and spore production (Fig. 1). As for the total-N of the mycelium, the lower the cultural temperature, the higher was the proportion of N-content (Table II).

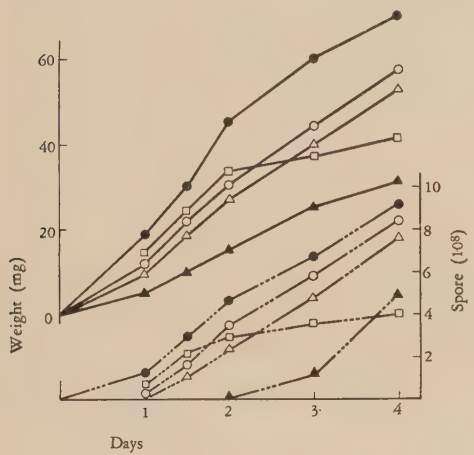


FIG. 1. Effect of Cultural Temperature on the Mycelial Weight and the Spore Production of *Asp. soyae* KS in the Paste Culture.

—▲— 20°C, —△— 25°C, —○— 30°C, —●— 35°C, —□— 40°C.

2. Effect of Moisture Content of Medium: The growth of *Asp. soyae* KS on the paste-medium whose initial moisture content was adjusted to 75, 65 and 50 per cent is shown in Fig. 2. The speed of mycelial growth as well as the increase of mycelial weight became greater as the moisture content of the medium became higher, but with regard to the spore production, the relation was just reverse, and the colour of spores on the medium of a less moisture content was more yellowish than against the more greenish colour on the medium of the higher moisture content.

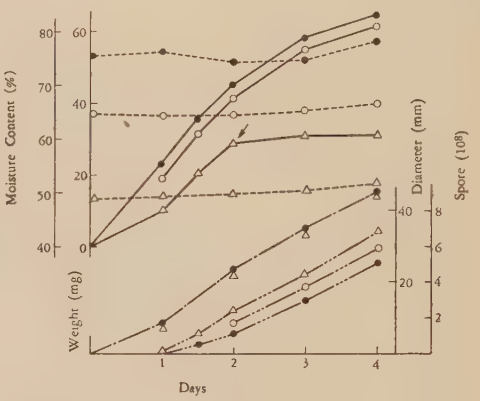


FIG. 2. Effect of the Initial Moisture Content on the Mycelial Growth and Weight and the Spore Production of *Asp. soyae* KS in the Paste Culture.

—●— 75% —————— mycelial weight
—○— 65% – – – – – spore
—△— 50% ——– —— mycelial growth
– – – – – – – moisture content
} being common to Fig. 2~Fig. 5.

A arrow line show the penetration of mycelium into the medium.

3. Effect of initial pH of Medium: On the medium whose initial pH was adjusted to

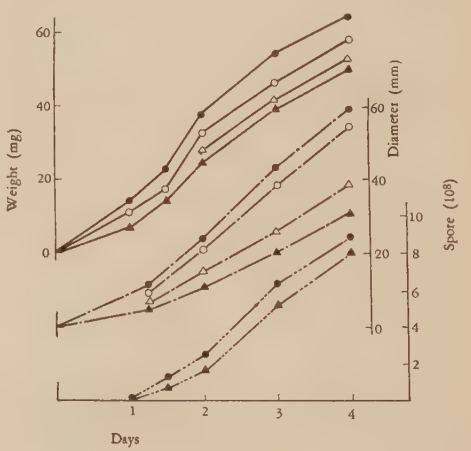


FIG. 3. Effect of Initial pH on the Mycelial Growth and Weight and the Spore Production of *Asp. soyae* KS in the Paste Culture.

—●— pH 5.0 —○— pH 6.0 —△— pH 7.0 —▲— pH 8.0

5,6,7 and 8, *Asp. soyae* KS was cultivated and the results are shown in Fig. 3. The speed of mycelial growth, the increase of mycelial weight and the unmber of spores produced at the lower initial pH were all superior to those at the higher pH in the range of the pH tested.

4. Comparison of Growth on three Kinds of Koji Materials: Growth on wheat bran, rice bran, copra meal were compared with each other from the view-point of production of mold protease, which will be described in the next report. The general constituents of four kinds of koji materials used are shown in Table III. With regard to the mycelial growth and the spore production, rice bran and wheat bran were almost equal in effectiveness, and copra meal was not so good as the others (Fig. 4). The mycelial weight of wheat bran was the highest among all phases of culture, copra meal following, though it was the lowest in the beginning, and that of rice bran became the lowest in the later-period of culture (Fig. 4).

5. Effect of C/N Ratio of Medium: To investigate the effect of the C/N ratio of medium, wheat bran as the C-rich material and defatted soy bean as the N-rich material were used. Paste media of several different C/N ratios

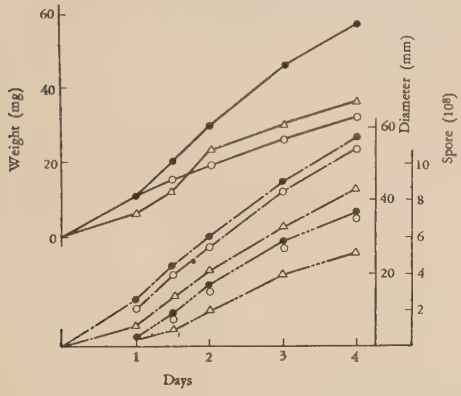


FIG. 4. Comparison of the Mycelial Growth and Weight and the Spore Production of *Asp. soyae* KS on Three Substrates in the Paste Culture.

—●— wheat bran —○— rice bran —△— copra meal

TABLE IV

EFFECT OF C/N RATIO OF THE PASTE-MEDIUM ON THE N-CONTENT OF THE MYCELIUM IN THE CULTURE OF 76 HOURS

	Composition of Medium				N-content
A:	wheat bran	10 g	defatted soy bean	0 g	5.25%
B:	〃	7.5	〃	2.5	6.08 〃
C:	〃	5	〃	5	6.42 〃
D:	〃	2.5	〃	7.5	7.11 〃
E:	〃	0	〃	10	7.70 〃

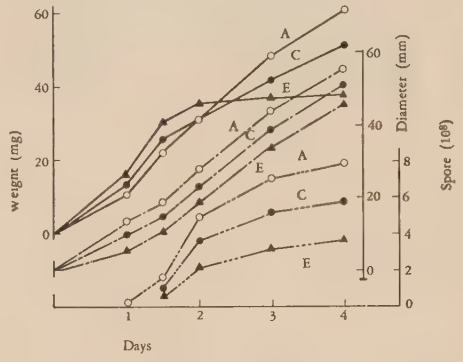


FIG. 5. Effect of the C/N Ratio of the Medium on the Mycelial Growth and Weight and the Spore Production of *Asp. soyae* KS in the Paste Culture.

A E: each medium have the same composition as in Table IV

TABLE III

GENEERAL CONSTITUENTS OF KOJI MATERIALS

Kind of koji material	Constituents					
	Water	crude carbohydrate	crude protein	crude fat	crude fibre	ash
wheat bran	10.14%	56.13%	11.83%	4.61%	8.29%	5.12%
rice bran	8.12 〃	32.79 〃	14.22 〃	5.33 〃	7.38 〃	28.70 〃
copra meal	14.81 〃	24.72 〃	19.47 〃	14.36 〃	13.80 〃	8.35 〃
defatted soy bean	10.12 〃	20.12 〃	50.27 〃	5.70 〃	6.21 〃	5.92 〃

were prepared by mixing these two materials as shown in Table IV. As seen in Fig. 5, the higher the C/N ratio, the larger mycelial growth as well as mycelial weight were, and the colour of spores became more greenish. But within 48 hours-culture, the low C/N ratio mycelia showed a slightly greater weight. The N-content of the mycelium kept pace with the N-content of medium as shown in Table IV.

DISCUSSION

The properties of koji profoundly depend upon the raw material used, and upon the chemical and physical environmental conditions under which koji-making are carried out, also upon the characters of the mold strain itself, which is studied in routine microbiological methods.

Since koji materials are used in the grain form, coarse ground form, bran form, or the mixed form of these, the factors which influence the properties of koji are too numerous and complicated to derive an exact conclusion on the function of each of these factors. It is, therefore, necessary to simplify the situation by some means. The paste-medium technique, as described adove, may be said to be the one which will meet such a request. By the use of this method employing *Asp. soyae* KS, the effects of cultural temperature, initial moisture content, initial pH, C/N ratio and kind of koji materials upon mycelial growth, mycelial weight, spore production and spore colour can be comparatively cleary investigated.

The author wishes to express his sincere thanks to Prof. K. Sakaguchi and Prof. Y. Asai for their kind advice and suggestions rendered throughout the course of this study, also Prof. H. Tamiya and Assist Prof. H. Iizuka for their advice on the use of the nylon.

The author also wishes to thank Dr. M. Mogi and Dr. N. Iguchi for their kindness for affording much facilities to accomplish this study.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 313~318, 1957]

Studies on Koji

Part II. Effects of Some Conditions of Medium on the Production of Mold Protease

By Kisirō Yamamoto

Noda Institute for Scientific Research

Received May 22, 1957

By employing the paste-medium technique, the effects of some conditions of medium on the protease production by *Asp. soyae* KS were investigated. The medium of initial pH of 7–8, initial moisture content of 50 per cent, high C/N ratio, and of rice bran, copra meal had each favorable influence on protease production. Protease which was produced in the paste culture was observed in the mycelium as well as in the medium, while in the case of liquid culture it was mostly secreted into the medium. The secreted protease in the paste-medium was found to difuse to the depth of 2.5 mm under the surface of the mycelium.

The production of mold protease has been intensively investigated by many workers from various view-points[1]. But to date no study was quantitatvely undertaken in the relation between growth and protease production of the mold, because, in the state of koji, mold mycelium and koji material could not be easily separated. In this paper the effects of cultural conditions on protease production by *Asp. soyae* KS, whose growth was investigated in the previous report[2], were studied in the mycelium as well as in the medium.

General cultural conditions such as initial pH, moisture content, and kind of koji materials are dealt in this paper, whilst the effect of temperature on the mold protease production will be reported in the next paper[3]. Moreover, by means of the paste-medium technique, the diffusion of mold protease into koji material in the paste culture was investigated, and compared with that of the liquid culture.

Since the number of mold proteases were at first recognized to be three by Kurono[4], and then four by Johnson[5], it has lately been observed that even a single strain of mold produces several kinds of protease. *Asp. soyae* KS was found to produce acid protease at the optimal pH of 3–4, and alkaline protease at the optimal pH of 7–10[6]. Moreover, in this report, it was found that *Asp. soyae* KS produces neutral protease[7] having resistance power against the potato-inhibitor[8]. Among these three kinds, alkaline protease was mainly investigated in this report, because it was mostly produced by this mold.

EXPERIMENTAL

1. Cultural Media

a) Paste-Medium: as that described in the previous paper.

b) Koji-medium: 5 g of koji materials was mixed 5 cc of distilled water, which was put into a flask of 150 cc, and sterilized for 30 minutes at 15 lb.

1) K. Yamamoto, lecture delivered at the Meeting of Protease Research No. 7, (1957).

2) K. Yamamoto, in press.

3) K. Yamamoto, in press.

4) K. Kurono, *Report of the Institute for Brewing*, **115**, 43 (1932).

5) M.J. Johnson, *Z. physiol. Chem.*, **224**, 163 (1934).

6) N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan*, **29**, 389 (1955).

7) T. Yasui, lecture delivered at the Meeting of J. Agr. Chem. Soc. Japan (1955).

8) K. Matsusima, *J. Agr. Chem. Soc. Japan*, **29**, 883 (1955).

c) Liquid-Medium[9]: a medium containing the following ingredients was chosen because a great deal of alkaline protease was observed to be produced by this mold cultured in this medium: 10 g of glucose, 4.2 g of peptone, 30 g of K_2HPO_4, 0.1 g of $MgSO_4$7aq, and 0.01 g $ZnCl_2$ in a 1–l medium, adjusted to pH 7, by a NaOH solution. A quantity of 50 cc of the liquid medium was taken into a 150 cc flask, and it was sterilized for 15 minutes at 10 lb.

2. Enzyme Preparation

a) Paste culture: The mycelium, cut with a borer of 20 mm in diameter, and washed well on the nylon-side, was mashed in a glass-mortar with 20 cc of water. By keeping the mashed mycelium for 4 hours at room temperature, enzyme solution A was gained as the filtrate. The filtrate obtained in the same way from the mycelium and the corresponding medium mashed together was referred to as enzyme solution B. Thus, the activity of the protease secreted into the medium was calculated by the activity of B minus A. Concerning the diffusion of mold protease into the medium, the enzyme solution was prepared as followings: the paste-medium that hollowed out in a cylinder-form was cut horizontally so that each layer might have the thickness of 2.5 mm, and protease was extracted with 20 cc of water from each layer.

b) Koji culture: Protease of each culture was extracted with 50 cc of water, by shaking sometimes for 4 hours, at room temperature.

c) Liquid: The filtrate of the culture was used for testing the activity of mold protease in the medium. The mycelial part was mashed with 20 cc of water in a glass-mortar, and the filtrate used as the enzyme solution.

3. Determination of Protease Activity

a) Alkaline protease: The method of Hagiwara[10] originated from Anson's method[11] was slightly modified, in order to be applicable to this test. Two cc of Hammersten milk casein solution (2 per cent, containing M/10 phosphate buffer of pH 7.0) and 1 cc of the enzyme solution were mixed, and the mixture was incubated at 30°C, at pH 7.0 for various periods i.e., 10 minutes for the koji extract, 30 minutes for the liquid preparation and three hours for the paste extract, respectively. After the addition of 5cc of 5 per cent trichloracetic acid the action of protease was stopped, the casein solution acted on by protease was obtained as the filtrate. One cc of the filtrate, 5 cc of 0.4 M Na_2CO_3 and 1 cc of Folin's reagent were well mixed. The mixture was kept for 20 minutes at 30°C and became violet-coloured. The colour density was measured at 660 mμ, with a Hitachi photo-electric spectrophotometer, and the value was translated into the optical density, which corresponds to the protease activity.

b) Neutral protease: one cc of Hammersten milk casein solution (4 per cent, containing M/5 phosphate buffer of pH 7.0), 1 cc of the potato-inhibitor prepared by Yasui's method[7], and 1 cc of the enzyme solution were mixed, and mixture was treated in the same manner as in the case of alkaline protease.

c) Acid protease: After 2 cc of Hammersten milk casein solution (2 per cent, containing M/10 lactate buffer of pH 3.5) and 1 cc of the enzyme solution extracted with M/10 lactate buffer of pH 3.5 in place of distilled water were mixed, the activity of acid protease was measured in the same manner as alkaline protease.

RESULTS

1. Determination of Neutral Protease

After various volumes of the potato-inhibitor solution and enzyme solution were incubated together, the activity of protease was measured in the usual manner. As a result, the activity of the neutral protease remained more or less showing resisting power against the potato-inhibitor (Table I). The activity of alkaline protease showed no resistance power to the potato-inhibitor, agreeing with Yasui's view[7].

2. Effect of Moisture Content of Medium

The mold protease production on the paste-medium whose initial moisture content was adjusted to 50, 65 and 75 per cent, was investigated. The protease activities both in the mycelium and in the medium of a low moisture content were found to be higher than those of a higher moisture content throughout all periods of culture.

Protease activities per dried mycelial weight of the lower moisture content were also higher than those of the higher moisture content.

Each of activity curves had one peak, and

9) K. Motonaga, read at the Meeting of J. Agr. Chem. Soc. Japan (1956).

10) B. Hagiwara, Kagaku-Jikken-Gaku, 207 (1953).

11) M.L. Anson, *J. Gen. Physiol*,. **22**, 79 (1938).

12) K. Kageyama and O. Sugita, *J. Ferm. Tech.*, **33**, 53 (1955).

TABLE I
RESISTANCE OF MOLD PROTEASE AGAINST THE POTATO-INHIBITOR

Constituents of pre-incubation						Protease activity (O.D.)
Potato-inhibitor	1 cc	Enzyme preparation	1 cc	Distilled water	0 cc	0.040
〃	0.5 cc	〃	〃	〃	0.5 cc	0.043
〃	0.05 cc	〃	〃	〃	0.95 cc	0.090
〃	0.025 cc	〃	〃	〃	0.975 cc	0.158
〃	none	〃	〃	〃	1 cc	0.449

P-II prepared by Yasui's method was used as the original solution of the potato-inhibitor. The pre-incubation was performed for 30 minutes at room temperature. After 1 cc of the pre-incubation and 2 cc of Hammersten milk casein solution (2 per cent, containing M/10 phosphate buffer of pH 7.0) were mixed, and incubated for 30 minutes at 30°C, protease activity was determined in the usual manner.

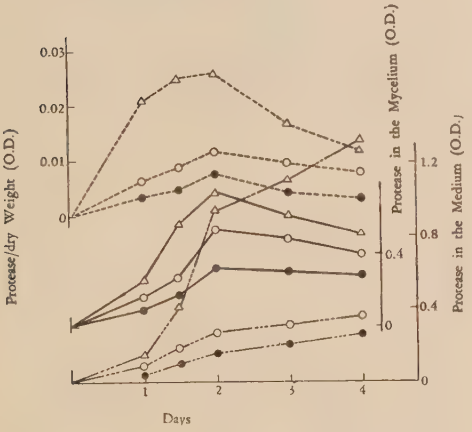


FIG. 1. Effect of the Moisture Content on the Production of the Mold Protease in the Paste Culture.

—△— 50%
—○— 65%
—●— 75%
——— protease in the mycelium
– – – – protease in the medium
– – – – – protease in the mycelium per mycelial weight
} being common to Fig. 1, 4, 6.

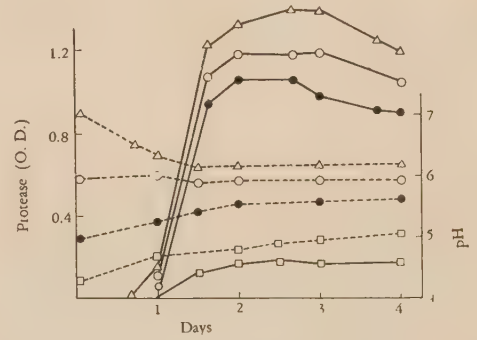


FIG. 2. Effect of Initial pH on the Production of the Mold Protease in the Wheat Bran Koji Making.

—△— pH 7.0
—○— pH 6.0
—●— pH 5.0
—□— pH 4.0
——— protease activity
– – – – pH changing
} being common to Fig. 2, 3

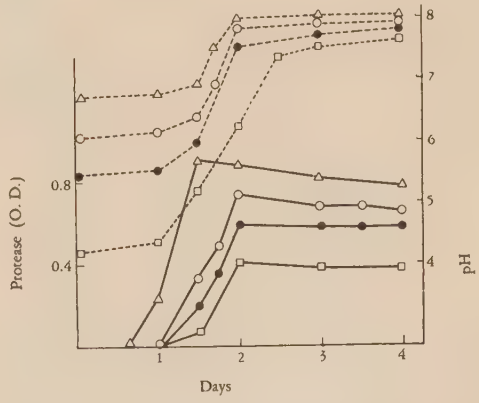


FIG. 3. Effect of Initial pH on the Production of the Mold Protease in the Defatted Soy Bean Koji Making.

after each peak, activity decreased alike (Fig. 1).

3. Effect of Initial pH of Medium

At first, the protease production of the wheat bran koji and soy bean flour koji whose initial pH were adjusted to 4–7 were investigated. As it is shown in Figs. 2-3, the higher the initial pH of medium, the higher is protease activity. At this time, a tendency was observed that during koji making the pH of the wheat bran koji approaches to 6.0, and the pH of the soy bean flour koji to 7.6. As for the paste-culture, the same tendency was observed as in koji (Fig. 4). Each pro-

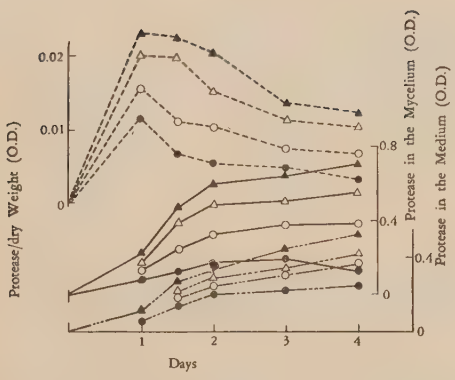


FIG. 4. Effect of Initial pH on the Production of the Mold Protease in the Paste Culture.

—▲— pH 8.0
—△— pH 7.0
—○— pH 6.0
—●— pH 5.0

tease activity in the mycelium per dried mycelial weight had one peak, which decreased gradually after the peak as is shown in Fig. 4.

4. Effect of Three Kinds of Koji Materials

The results of the investigation conducted on protease production in three kinds of koji making are shown in Fig. 5. The mold produced more protease than in the wheat bran koji in both the rice bran koji and the copra meal koji. According to the investigation of the paste culture, protease activity in the mycelium were all similar among the three kinds of materials, but protease in the medium of rice bran and copra meal were much more active than that in the medium of wheat bran (Fig. 5).

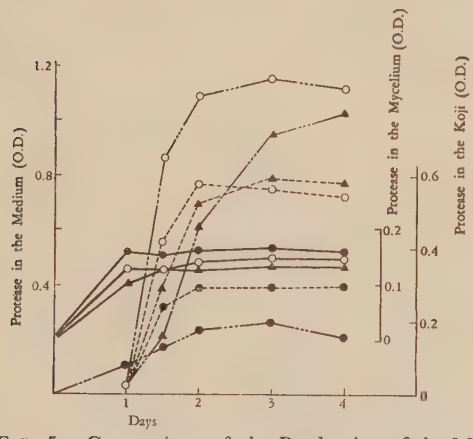


FIG. 5. Comparison of the Production of the Mold Protease between Three Substrates in the Paste Culture and in the Koji Making.

—▲— copra meal
—○— rice bran
—●— wheat bran
——— protease in the mycelium
— - - - protease in the medium
- - - - - protease in the koji

5. Effect of C/N Ratio of Medium

Protease production in the paste culture was investigated by mixing the wheat bran and soy bean flour. The more the proportion of the wheat bran (C-rich medium), the more protease was produced.

But protease activity per dried mycelial weight was maxium when quantity of wheat bran was equal to that of soy bean flour (Fig. 6).

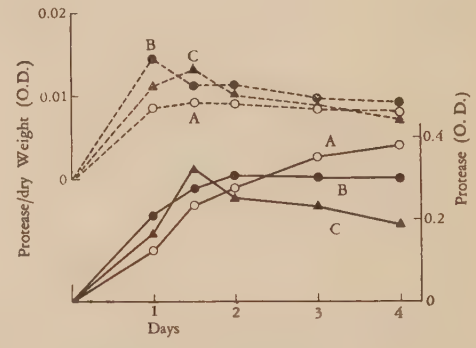


FIG. 6. Effect of C/N Ratio on the Production of the Mold Protease in the Paste Culture.

A: wheat bran 10 g plus soy bean flour 0
B: 〃 5 g 〃 5 g
C: 〃 0 〃 10 g

6. Distribution of Protease between the Mycelium and the Medium

The distribution of three kinds of protease produced by the mold was investigated, both in the mycelium and in the medium during the wheat bran culture. As shown in Table II, three kinds of protease produced by this mold presented more mycelium than in the medium throughout all cultural phases.

7. Diffusion of Protease secreted into the Medium

The medium horrowed out in a cylinder-form during the paste culture was cut hori-

TABLE II
DISTRIBUTION OF PROTEASE ACTIVITIES BETWEEN THE MYCELIUM AND THE MEDIUM DURING PASTE CULTURE

Kind of Protease	Presentation	Protease activity (O.D.)		
		24 hrs	48 hrs	72 hrs
Alkaline	in the mycelium	0.06	0.115	0.14
	in the medium	0.025	0.080	0.11
Neutral	in the mycelium	0.065	0.16	0.185
	in the medium	0.030	0.065	0.060
Acid	in the mycelium	0.12	0.135	0.145
	in the medium	0.035	0.045	0.050

When the activity of alkaline protease is compared with that of other kinds of protease, the value is increased 10 times.

zontally so that each layer might be 2.5 mm-thickness. Protease activity in each layer was then investigated. The result showed that the greater part of the protease secreted into the paste-medium are detected right under the mycelium, and a small amount is found in the medium in the depth of 2.5 mm (Fig. 7).

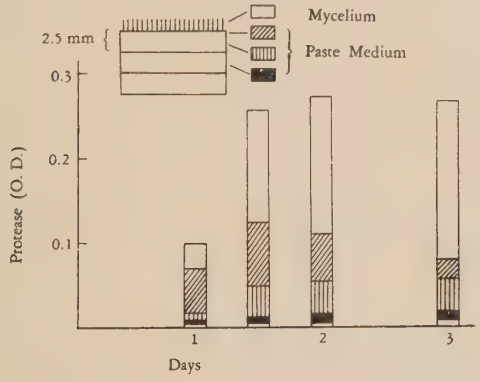


FIG. 7. Diffusion of the Mold Protease Secreted into the Medium in the Paste Culture.

DISCUSSION

In this test it was clearly demonstrated that the moisture content of koji materials influences mold protease production. The growth of this mold was better on the medium of a higher moisture content than those of lower moisture content as shown in the previous paper[2]. However, circumstances favorable for protease production were observed to be adverse to growth of the mold. In the case of liquid culture[9,13,14], more protease was produced by the mold in either a neutral or alkaline medium than in a acidic medium, and the same results was also obtained with koji here and another test[15]. As the results of these investigations, it was supposed that the less protease activity at a low pH was due to the fact that the protease secreted into the medium might be destroyed by the effect of pH of the medium.

But according to the test of the paste culture, it was obvious that the pH of the medium had some influence on protease production by this mold. It is a well known fact that there are differences in protease activities between various koji makings. It was also recognized here and from other tests[19,20] that rice bran and copra meal are superior to wheat bran with regard to mold protease production. The investigation in this paper gives some suggestion that the components of the former two koji materials have better influence than the latter either on the protease secretion or on the stability of the secreted protease. Regarding that the C/N ratio of the medium has influence upon the mold protease production, investigations with liquid culture[14,21] were carried out, and Nakano et al., reported that the more the proportion of wheat bran, the more protease is produced in koji making from wheat bran plus soy bean flour. This tendency was also observed with paste culture of *Asp. soyae* KS. The fact that protease activity per dried

13) M.E. Maxwell, *Aust. J. Sci. Ser. B. Biol. Sci.*, **5**, 42 (1952).
14) T. Irie, *J. Agr. Chem. Soc. Japan*, **28**, 550 (1953).
15) M. Matsuyama, *J. Ferm. Tech.*, **28**, 149 (1950).
16) G. Kita, *Kogyo-Kagaku-Zassi*, **12**, 399 (1910).
17) M. Itō, *J. Ferm. Tech.*, **4**, 394 (1927)
18) K. Oshima, *J. Ferm. Tech.*, **1**, 579 (1924).
19) N. Inoue and K. Kusaka, *J. Ferm. Tech.*, **30**, 414 (1952).
20) K. Kageyama and O. Sugita, *J. Ferm. Tech.*, **33**, 109 (1955).
21) M. Nakano and H. Ebine, *Report of the Food Research Institute*, **No. 8**, 163 (1953).
22) M. Nakano and H. Ebine, *Report of the Food Research Institute*, **No. 8**, 169 (1953).

TABLE III
PRODUCTION OF MOLD PROTEASE IN THE LIQUID CULTURE

Cultural time	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
Dried mycelial weight	372 mg	452 mg	530 mg	584 mg	713 mg
Protease activity in the mycelium	0.68	0.80	0.70	0.66	0.60
Protease activity in the medium	22.0	29.7	37.2	31.05	32.25

Values show total activity (O.D.) in the mycelium and in the medium.

mycelial weight reached its maximum when the quantity of mixed wheat bran was equal to that of soy bean flour was significant as a favorable condition of koji making. It was observed that the protease produced by the mold existed mostly in the mycelium, thus making a clear contrast to the case of the liquid culture where it existed mostly in the medium (Table III).

The author wishes to express his sincere thanks to Prof. K. Sakaguchi and Prof. Y. Asai for their kind advice and suggestions rendered throughout the course of this study, and also to Assist. Prof. B. Maruo for his advice.

Thanks are also due to Dr. M. Mogi and Dr. N. Iguchi for their kindness for affording much facilities to carry out this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 319~324, 1957]

Studies on Koji

Part III. Effects of Cultural Temperature on the Production of Mold Protease

By Kisirō Yamamoto

Noda Institute for Scientific Research

Received May 22, 1957

In the preparation of koji from various raw materials, it was recognized that the lower the cultural temperature, the more the three kinds of protease are produced by *Asp. soyae* KS. In the paste-culture, the amounts of production of mold protease both in the mycelium and in the medium were also more at lower cultural temperatures than at higher ones. The low temperature should be applied at a period which coincides with that of spore-production because at such period the mold protease production is observed to be maximum. The limit of the practical low temperature was about 12°C, which was also the limit to growth of the mold tested.

With regard to the heat-resistance of mold protease, there have been many studies since Ōshima's report[1] (1923), in relation to the pH-resistance of mold protease. However, concerning the influence of cultural temperature on the mold protease production, few papers have appeared since Oana[2] reported the relation between the protease activity and various temperatures in rice koji making. Maxwell[3] and Petrenko et al[4]., observed that the optimum temperature for the protease production was 22°C in case of *Asp. flavus* and 18°C in case of *Asp. terricolla*, respectively.

Kalashmikov et al[5]. (1955), reported that the preparation obtained by culturing *Asp. oryzae* at 24°C, for 48 hours had a proteolytic activity of 1.5–3 times as potent as one obtained at 30°C. Suzuki et al[6]. (1956), investigated the production of mold protease at three different cultural temperatures ——33°C, 38°C and 43°C—— in rice koji making and recognized that the optimum cultural temperature for production was 33°C.

In this paper the effect of cultural temperature on protease production by *Asp. soyae* KS during several koji makings, and that in the mycelium and in the medium during paste culture is investigated. Besides these, the effect of the change of temperature on the mold protease production during the culture-period and the lower-limit of temperature enabling the mold to produce protease are invesigated.

EXPERIMENTAL

1) Strain tested

Asp. soyae KS was chosen, as in the previous paper[7].

2) Cultural Media

Each medium for the paste culture, koji and the liquid culture was prepared in the same method as described in the previous report[7].

3) Enzyme Preparation

According to the difference of methods of culture and in qualities of the protease produced, various

1) K. Ōshima, *J. Ferm. Tech.*, **1**, 579 (1923).

2) H. Oana, *J. Ferm. Tech.*, **14**, 1 (1936).

3) M.E. Maxwell, *Aust. J. Sci. Res. Ser. B. Biol. Sci.*, **5**, 42 (1952).

4) I.N. Vinogradova and I.P. Pltonova and V.A. Petrenko, *Mikrobiologia*, **21**, 692 (1952): *C.A.*, **47**, 7007 (1953).

5) E. Ya. Kalashmikov and D.B. Lifshts and B.M. Lerintan, *C.A.*, **50**, 6577 (1956).

6) M. Suzuki and Y. Nunokawa, *J. Soc. Brew. Japan*, **51**, 58 (1956).

7) K. Yamamoto, in press

enzyme solutions were prepared in the same way as described in the previous paper[7].

4) Measurement of Protease Activity

Activity of each the three kinds of protease produced by this mold were measured in the same way as in the previous report[7].

RESULTS

1. The Effect of Cultural Temperature on the Production of Three Kinds of Protease during Koji-Making

The effect of several levels of cultural temperature (20–35°C) on the production of acid, neutral and alkaline protease in koji-making from various koji materials—wheat bran, rice bran, copra meal, defatted soy bean and rice—were investigated. As shown in Figs. 1–5, the alkaline protease had each a characteristic curve corresponding to various koji materials, but the influence of cultural temperature on protease production were all similar in the respect that is, the lower the cultural temperature, the more powerful the protease activity becomes. The wheat bran koji, whose initial pH was adjusted to the range 5 to 7 by means of K_2HPO_4, also produced more protease at low cultural temperatures than at higher ones (Figs. 6, 7). As for the other two kinds of protease, the same tendency as this was observed.

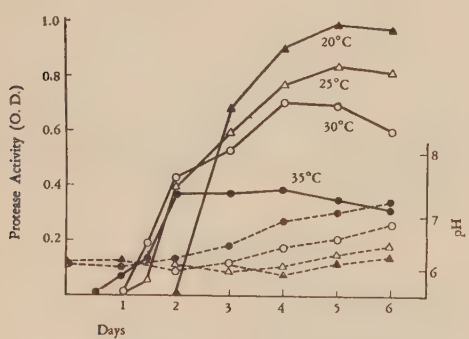


FIG. 1. Effect of the Cultural Temperature on the Production of the Alkaline-protease in the Wheat Bran Koji Making.

—●— 35°C
—○— 30°C
—△— 25°C
—▲— 20°C
—— protease activity
---- change of pH
} being common to Fig. 1~12.

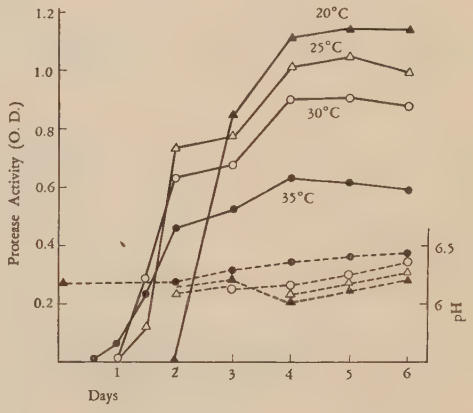


FIG. 2. Effects of the Cultural Temp. on the Production of the Alkaline-protease during the Rice Bran Koji Making.

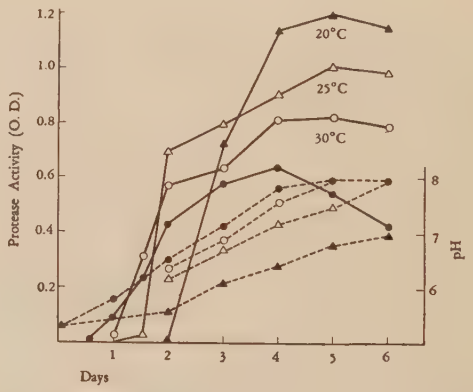


FIG. 3. Effect of the Cultural Temp. on the Production of the Alkaline-protease in the Copra Meal Koji Making.

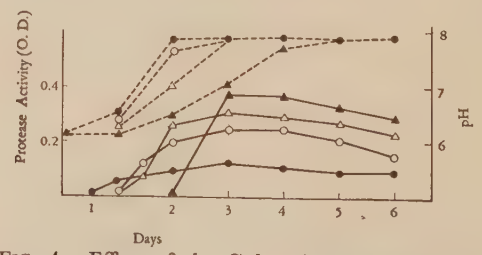


FIG. 4. Effect of the Cultural Temp. on the Production of the Acid-protease in the Defatted Soy Bean Koji Making.

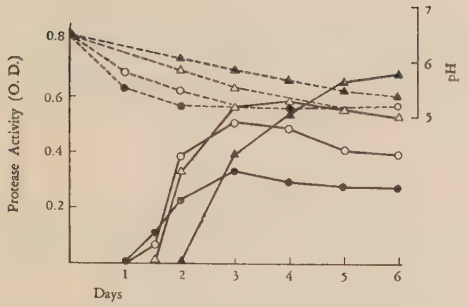


FIG. 5. Effects of the Temp. on the Production of the Acid-protease during the Rice-koji Making.

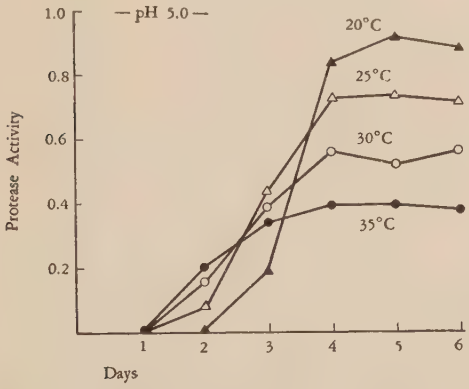


FIG. 6. Effects of the Temp. and pH on the Production of the Alkaline-protease during the Wheat Bran Koji Making.

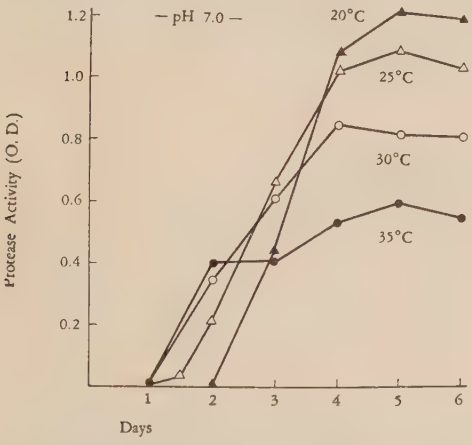


FIG. 7. Effects of the Temp. and pH on the Production of the Alkaline-protease during the Wheat Bran Koji Making.

2. The Effect of Cultural Temperature on the Ratio of the Three Kinds of Protease Produced in Koji-Making

Activity of each of the three kinds of protease produced by *Asp. soyae* KS in the wheat bran koji-making at different cultural temperature were expressed in percentage of the total protease activity. As it is shown in Table I, the activity of alkaline protease was the most remarkable among the three, and the ratio of the three kinds of protease remained almost constant throughout all phases of culture.

TABLE I
EFFECT OF CULTURAL TEMPERATURE ON THE RATIO OF ACTIVITIES OF THE THREE KINDS OF MOLD PROTEASE IN THE PASTE CULTURE

Cultural temperature	Kind of protease	Cultural time 48 hrs	72 hrs	120 hrs
20°C	acid	8%	5%	4%
	neutral	8 〃	10 〃	10 〃
	alkaline	84 〃	87 〃	86 〃
30°C	acid	6 〃	6 〃	5 〃
	neutral	8 〃	8 〃	10 〃
	alkaline	86 〃	87 〃	85 〃
35°C	acid	6 〃	4 〃	4 〃
	neutral	8 〃	10 〃	8 〃
	alkaline	86 〃	86 〃	88 〃

Values indicate the ratio of the activity of each kind of protease per total activity of the three kinds of protease at each period of culture.

3. The Effect of Cultural Temperature on the Mold Protease Production in the Mycelium and in the Medium in the Paste Culture

By employing the paste culture method, the effect of cultural temperature on mold protease production was investigated. The lower the cultural temperature, the higher the mold protease activity in the mycelium became, as was in the case of koji-making (Fig. 8).

At low temperatures the protease activity in the mycelium on the dry mycelial weight was still more remarkable than at higher temperature, because 35°C was optimum for

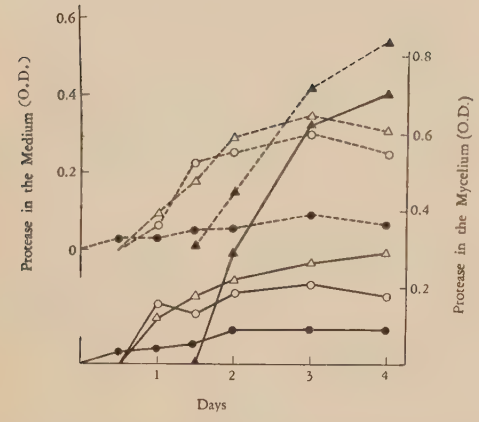


FIG. 8. Effect of Cultural Temperature on the Production of the Mold Protease in the Paste Culture.

——— - protease activity in the mycelium.
- - - - - protease activity in the medium.

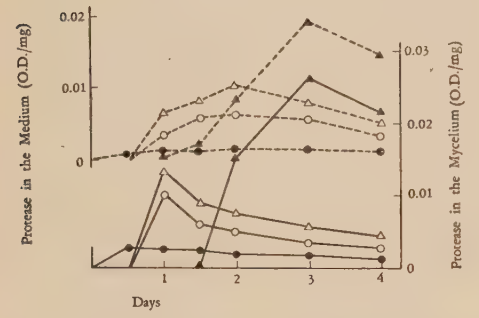


FIG. 9. Effect of Cultural Temperature on the Production of the Mold Protease per Dried Mycelial Weight in the Paste Culture.

the growth of this mold (Fig. 9). Also, each curve of the protease activity in the mycelium based on the dry mycelial weight assumed similar peak. Protease activity in the medium showed the same tendency as in the case of mycelium, except that the peak of activity of the medium appeared a little later than that of the mycelium (Figs. 8, 9).

4. The Effect of the Change of Cultural Temperature on Mold Protease Production

If the cultural temperature was changed from 20°C to 35°C at the time when the protease activity was increasing, the final activity proved always to be lower than that in the case when cultural temperature was left unchanged at 20°C, but higher than in the case when it was kept at 35°C from the beginning (Table II). The same can be said with both the mycelium and the medium. Next, if the cultural temperature was changed from 35°C to 20°C after mold protease production became maximum, the activity of mold protease slightly increased than when its cultural temperature was left unchanged at 35°C, but still did not reach to the level of activity as when it was kept at 20°C from the beginning. These phenomena were common to both mycelium and medium protease. But if the cultural temperature was lowered down from 35°C to 20°C before the protease production reached its maximum rate, the protease could in the end reach its maximum

TABLE II
EFFECT OF THE CHANGE OF CULTURAL TEMPERATURE ON THE MOLD PROTEASE PRODUCTION IN THE PASTE CULTURE

Cultural temperature	Presentation	Cultural-time 36 hrs	48 hrs	72 hrs	96 hrs
20°C	in the mycelium	0.10	0.25	0.37	0.32
	in the medium	0.08	0.15	0.25	0.27
35°C	in the mycelium	0.05	0.06	0.05	0.05
	in the medium	0.05	0.05	0.04	0.04
from 20°C to 35°C (at the phase of 48 hrs)	in the mycelium			0.19	0.12
	in the medium			0.08	0.07
from 35°C to 20°C (at the phase of 24 hrs)	in the mycelium		0.10	0.19	0.20
	in the medium		0.09	0.17	0.15

Values indicate optical density as protease activity.

activity attainable, when cultural temperature was kept at 20°C from the very beginning (Table III). The culture at 35°C reached its maximum activity in about seventeen hours, when the mold was beginning to produce spores most vigorously.

TABLE III
EFFECT OF CHANGE OF CULTURAL TEMPERATURE ON THE MOLD PROTEASE PRODUCTION IN THE PASTE CULTURE

Phase+ of culture at 35°C	Cultural-time++			
	36 hrs	48 hrs	72 hrs	120 hrs
0 hr.	0.025	0.211	0.413	0.492
3 〃	0.210	0.306	0.400	0.495
6 〃	0.301	0.351	0.424	0.475
9 〃	0.276	0.306	0.431	0.484
12 〃	0.245	0.292	0.415	0.496

+shows the phase when cultural temperature was changed from 35°C to 20°C.
++shows the total cultural time both at 35 and 20°C.
Values indicate optical density as protease activity.

5. Minimum Cultural Temperature for the Maximum Mold Protease Production

As is shown in Fig. 10, the mold protease production was markedly higher at 15°C than at 20°C, and slightly higher at 13°C than 15°C. But the lower the cultural temperature, the later protease activity reached its maximum because of the slower moldgrowth. At 10°C this mold did not grow nor sporulate, consequently, protease activity did not increase at all.

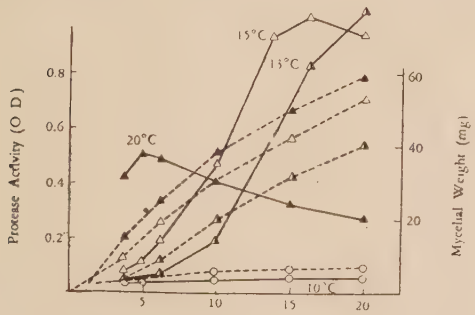


FIG. 10. Effect of the Lower Cultural Temperature on the Growth and the Protease Production of the Mold in the Paste Culture.
——— protease activity
- - - - - dried mycelial weight

DISCUSSION

The recent studies[3, 4, 5, 8] on mold protease production have suggested that the optimum temperature for the protease production might exist at about 20°C. In this investigation, it was recognized that the lower the cultural temperature, the higher mold protease activity becomes so far as the mold could grow, and that the lower the cultural temperature, the later protease activity reaches its maximum. It was also observed that these phenomena were common to various koji, pH of medium and the three kinds of protease. Each curve representing protease activity per dry mycelial weight had one peak and activity decreased behind it. Besides, the activity of enzyme solution decreased if the enzyme preparation was kept at several temperatures (Fig. 11). Accordingly, it is supposed that the protease activity at each stage of koji-making will show a balance between the production and the de-

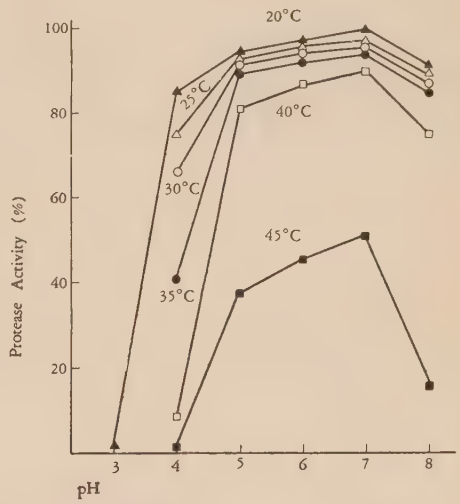


FIG. 11. Effect of the Temp. and pH on the Stability of the Mold Protease.

The protease activity of each enzyme preparation being kept at various pH and temperature for 24 hours were determined against to that of the original preparation.

8) M. Mogi, N. Iguchi, H. Yoshida and K. Yamamoto, in press

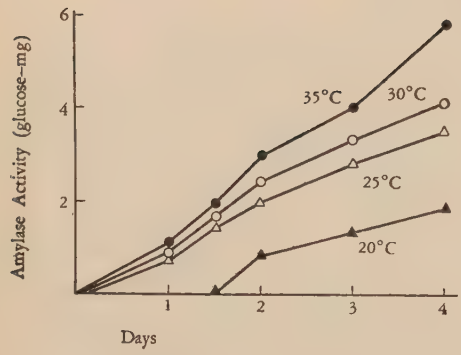


FIG. 12. Effect of the Cultural Temperature on the Production of the Mold-amylase in the Paste Culture.

struction of mold protease. In the case of higher temperatures, a great part of protease secreted out of the mycelium would be destroyed, and accordingly a smaller quantity of protease would be determined. But it is also supposed that cultural temperature would immedate have influence upon the mold protease production in the mycelium, because the protease activity in the mycelium was higher at low temperatures, and that the low temperature would have favorable influence on protease production at a phase when the mold was vigorously producing protease. Nomura et al[9]., suggested that the amylase production of *Bac. subtilis* competed with normal protein synthesis for growth of this microbe. It is supposed that the mold protease production is also an abnormal biosynthesis, as suggested by Nomura et al., because the mold produced little protease in the phase of hyphae-growing, and much more in the spore producing-phase. Further, it is supposed that the lower the cultural temperature, the stronger the abnormal biosynthesis becomes. But on the other hand, it will be supposed that the mold protease produced in the mycelium may get inactivated in the mycelium at higher temperatures as well as in the medium, because the mold amylase, which is also an exoenzyme as protease, was produced keeping pace with the mold growth (Fig. 12). These suppositions will be examined later on.

9) S. Nomura, J. Hosoda, B. Maruo and S. Akabori, lecture delivered at the Meeting of Symposium of Enzyme-Chemistry, (1956).

The author wishes to express his sincere thanks to Prof. K. Sakaguchi and Prof. Y. Asai for their kind advice and suggestions rendered throughout the course of this study. The author also wishes to thank Dr. M. Mogi and Dr. N. Iguchi for their kindness in affording much facilities to carry out this study, and to T. Kuramochi for his kind assistance.

Maltase Action of Bacterial α-Amylase

Sir;

α-Amylase has been reported as being devoid of maltase action, although there is an exceptional report by Myrbäck[1] stating that salivary amylase exhibits maltase activity. Recently, however, α-amylase of *Bac. subtilis* var. *amyloliquefaciens* Fukumoto, which was reported to exhibit no maltase action even in a quite unpurified state[2,3], was found to hydrolyse maltose, provided that an extremely large amount of the enzyme is used.

The crystalline α-amylase obtained from the bacteria by the author's method which was recrystallized six times[4] and "Difco" maltose were used as the enzyme and substrate, respectively. Paper partition chromatography revealed that the enzyme contained no sugar and also that substrate contained no other sugar than maltose. To 1 ml of the solution containing 1 mg of maltose was added 1 ml of amylase solution, in which the amount of the enzyme was doubled in sequence from about 0.128 to 8.2 mg per ml. In a certain condition of hydrolysis of starch, 1 mg of the enzyme has been known to produce about 1.8 g of reducing sugar calculated as maltose in the course of five min. at 40°C[4]. In the present work, the amylase was used immediately after the crystals of the enzyme were dissolved in dilute sodium hydroxide solution. Phosphate buffer, M/100 in the final concentration, was used in the experiment in order that a good separation of the enzyme would be obtained by the procedure described later on. The pH of the reaction mixture was in the range 6.4 to 6.8, the value increasing with enzyme concentration. After incubating the reaction mixture for six to twenty-eight hrs. at 37°C, two volumes of alcohol were added, and the supernatant solution, after concentration to about 0.3 ml, was subjected to paper partition chromatography. As control runs, solutions containing "Difco" maltose, glucose, and the enzyme alone, respectively, were treated in the same way. The chromatography was performed by the ascending method using "Toyo" filter paper No. 131 and isopropanol : acetic acid : water (68 : 10 : 22) as the solvent. The chromatogram was developed by spraying with aniline hydrogen phthalate and heating to 100°C for 15 min.[5]. As shown in Fig. 1, the paper chromatogram that resulted from the reaction mixture to which over 2.05 mg of the enzyme had been added, clearly revealed that the greater part of maltose was hydrolyzed to glucose. On the other hand, the reaction mixture which contained less than 0.256 mg of the enzyme showed no formation of glucose, even after twenty-eight hrs. of incubation. In the solutions of maltose and of amylase, incubated as the controls respectively, no glucose was detected. With the crude amylase which was obtained only by salting out, followed by dialysis, from the culture filtrate of the amylase-producing bacteria, of which the coloring matter had been removed by an ion-exchange resin method[6], the maltase action was also observed at the amylase activity almost equal to that of the crystalline enzyme that exhibited the action.

1) K. Myrbäck and E.W. Willstedt. *Arkiv Kemi*, **6**, 465 (1953). *C.A.*, **48**, 6490 (1954).
2) J. Fukumoto, *Ann. Rep. Osaka Mun. Res. Inst. Technol.*, No. 9 (1943).
3) B. Hagihara, *Ann. Rep. Scient. Works. Fac. Sci. Osaka Univ.*, **2**, 35 (1954).
4) T. Yamamoto, This Bulletin, **19**, 121 (1955); **29**, 188 (1956).
5) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948); *Nature*, **164**, 443 (1949).
6) J. Fukumoto, T. Yamamoto and K. Ichikawa; to be published.

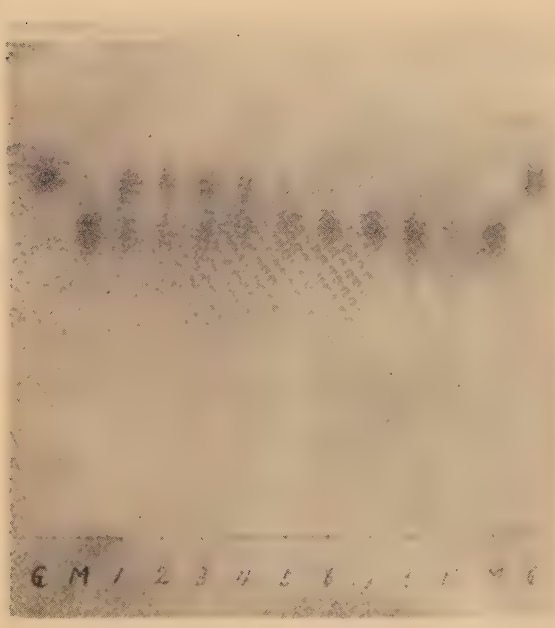


FIG. 1. Multiple Ascent Paperchromatogram of Maltose Digests by Bacterial α-Amylase.

Digests, No.	Incubation period, hrs.	Amylase, mg
1	28	8.20
2	28	2.05
3	6	8.20
4	6	2.05
5	28	0.513
6	28	0.256
7	6	0.513
8	6	0.256

G, glucose
M, maltose
E, amylase (8.2 mg, incubated for 28 hrs.).

From the result that the maltase action of bacterial α-amylase was exhibited depending more on the amount of the enzyme rather than on the period of incubation of the reaction mixture, it does not seem that the maltase action was due to some other enzyme contaminated in the amylase. The phenomenon described above might have some relation to the facts that the hydrolysis degree of starch by α-amylase differs considerably, according to the amount of the enzyme added[7~12] and that maltose is a non-competitive inhibitor of α-amylase[9,10,12]. However, the solution of the problems described above is left for the future.

The author wishes to express his sincere thanks to Prof. I. Yamasaki of Kyushu University and Prof. J. Fukumoto of Osaka City University, for their instructive suggestions. He is also indebted to Mr. Y. Hashimoto, for his assistance in this work.

Takehiko YAMAMOTO

Institute of Polytechnics,
Osaka City University

Received May 28, 1957

7) R.B. Alfin and M.L. Caldwell, *J. Am. Chem. Soc.*, **70**, 2534 (1948).
8) S. Schwimmer and A.K. Balls, *J. Biol. Chem.*, **179**, 1063 (1943).
9) S. Schwimmer, *ibid.*, **186**, 181 (1950).
10) K. Meyer et W.F. Gonon, *Helv. Chim. Acta*, **39**, 294 (1951).
11) S. Akabori et al., Delivered at the Meeting of "Symposia on Biochemistry of Starch", Fukuoka, Japan, Nov. 1952.
12) J. Fukumoto, T. Yamamoto and K. Ichikawa, *Symposia on Enz. Chem.*, **8**, 40 (1953).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 327~328, 1957]

Biochemical Studies on "Bakanae" fungus

Part XXXX. Chemical Structure of Gibberellins. X

Sirs:

In this communication, the further evidences are presented which make sure of the assumption proposed in our previous paper[1] on the relation between gibberellin A_1 and gibberellic acid.

As already described, methyl gibberellate gives two kinds of compounds by the catalytic hydrogenation with Adams' catalyser, uptaking 1.8 moles of hydrogen. One is dihydrogibberellin A_1 methyl ester, a mixture of two epimers, in 15–20% yield and the other is a hydrogenolysis product, $C_{20}H_{28-30}O_6$, in 60% yield.

Now, when free gibberellic acid is subjected to the same catalytic reduction, the following four products are separated through the partiton chromatography of silica gel:

1) an undetermined compound, d.p. 176–80°, in less than 5% yield.

2) monocarboxylic acid, $C_{19}H_{26}O_6$, d.p. 270–2°, in 35–40% yield, which is identical with dihydrogibberellin A_1, a mixture of two epimers, being confirmed by infrared spectrum and mixed melting point.

3) dicarboxylic acid, $C_{19}H_{28}O_6$, d.p. 290–5°, in 20% yield, which is a hydrogenolysed product.

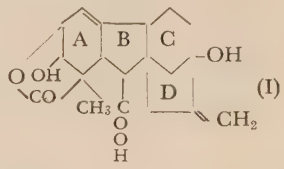
4) dicarboxylic acid, $C_{19}H_{28}O_6 \cdot 1/2\ H_2O$, d.p. 184–6°, in 30% yield, which is a hydrogenolysed product.

The fact that dihydrogibberellin A_1 is obtained in a fairly good yield from gibberellic acid supports our assumption concerning the relation between gibberellin A_1 and gibberellic acid.

Moreover, when gibberellin A_1 methyl ester and methyl gibberellate are treated with an excess of lithium aluminum hydride in boiling tetrahydrofuran for 24 hours, $C_{19}H_{32}O_5$ (A_1 Li product), m.p. 208–10°, from gibberellin A_1 methyl ester and $C_{19}H_{30}O_5$ (G. acid Li product) m.p. 208–10°, from methyl gibberellate are obtained, respectively. On hydrogenation with Adams' catalyser, the A_1 Li product absorbs no hydrogen, the original substance being recovered, and the G. acid Li product absorbs 1.5 moles of hydrogen, yielding the identical substance with the A_1 Li product.

These evidences lead us to the conclusion that gibberellic acid has the same configuration with gibberellin A_1, except that gibberellic acid has one more carbon-carbon double bond besides exocyclic methylene in the D ring, the existence of such a methylene group in gibberellin A_1 and gibberellic acid being reported in previous communication[2,3].

As the close similarity of structure of gibberellin A_1 and gibberellic acid is already confirmed, we intended to elucidate the structure of gibberellic acid, on which the structure (I) was proposed by Cross et al. tentatively. We have now reached a different conclusion from Cross' structure of ring A, especially the position of the lactone ring.



(I)

A_1 Li product and G. acid Li product do not consume periodate in either alkaline or acidic condition for 24 hours. These results

1) H. Kitamura, Y. Seta, N. Takahashi and Y. Sumiki: *Bull. Agr. Chem. Soc. Japan*, **21**, 71 (1957).

2) Y. Seta, H. Kitamura, N. Takahashi and Y. Sumiki: *Bull. Agr. Chem. Soc. Japan*, **21**, 73 (1957).

3) B.E. Cross, J.F. Grove, J. MacMillan and T.D.C. Mulhollan: *Chem. and Ind.*, **36**, 954 (1956).

clearly indicate that lactone alkyl oxygen is not located at the vicinal position to the original secondary hydroxyl group in A ring.

On the other hand, on alkali treatment* of gibberellin A_1, dihydrogibberellin A_1, isogibberellin A_1 and gibberellic acid, each of them consumes two moles of alkali. Then, the solution back-titrated to pH 8–9 was treated with potassium dimesoperiodate solution at the same pH.

In this experiment, both dihydrogibberellin A_1 and isogibberellin A_1[4] which is an isomer obtained by treating gibberellin C with alkali, do not consume periodate for a period of 24 hours. However, gibberellin A_1 consumes about one mole of periodate rather slowly while gibberellic acid consumes more than 4 moles of periodate very rapidly. We, therefore, examined the change caused by alkali treatment under the same condition* as described above, excluding periodic acid oxidation. The main products recovered by acidifying the alkali treated solution of gibberellin A_1, are gibberellin A_1 in 10% yield and an isomer of gibberellin A_1 in 50% yield. The latter is a different compound from pseudogibberellin A_1 and does not consume periodate under the above condition. In connection with the fact that dihydrogibberellin A_1 and isogibberellin A_1 do not consume periodate, it might be considered that a small, not the main portion of the alkali treated product, which cannot be isolated in a pure state through the partition chromatography of silica gel, consumes periodate.

While, on the same alkali treatment and acidification, gibberellic acid gives amorphous dicarboxylic acid, $C_{19}H_{26}O_8$, d.p. 152–4°, which readily consumes periodate in acid and alkaline conditions. Moreover, this substance absorbs only one mole of hydrogen, not two moles, by catalytic hydrogenation. The elementary analytical datum shows that two moles of water are added to the original formula, one of them being used in hydrolysis of the lactone ring. The result of catalytic reduction shows that one of the two double bonds present in gibberellic acid is absent in amorphous dicarboxylic acid. Consequently, we conclude that dicarboxylic acid is not a simple hydrolysis product of the lactone ring but a secondarily changed product——perhaps hydration of the double bond——and thus, the newly introduced hydroxyl group is vicinal to the lactone alkyl oxygen or original secondary hydroxyl group.

With regard to the position of the original secondary hydroxyl group in A ring, this will be reported in the coming near future.

We are indebted to Kyowa Fermentation Co., Takeda Pharmaceutical Co., Lederle Laboratories and Elli Lily Co. for generously supplying us the precious gibberellin A_1 and gibberellic acid.

Nobutaka TAKAHASHI
Yasuo SETA
Hiroshi KITAMURA
Yusuke SUMIKI

Department of Agricultural Chemistry
Faculty of Agriculture
University of Tokyo, Tokyo

Received July 23, 1957

*This condition is more vigorous than that in the case of obtaining pseudogibberellin A_1 from gibberellin A_1, and under the latter condition, the lactone ring of gibberellin A_1 is not opened.

4) N. Takahashi, Y. Seta, H. Kitamura, A. Kawarada and Y. Sumiki: *Bull. Chem. Soc. Japan*, **21**, 75 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 5, p. 329~331, 1957]

Infrared Spectrophotometric Determination of Traces of Parathion in Water

Sir:

In the previous communication[1], two methods for the microdetermination of parathion in water (on a scale of 10 γ or less as the total amount) were presented. As the total amount of parathion treated in those methods is very small, it is difficult to perform the identification of parathion through infrared spectroscopy There is, however, a keen need for specificity in determination of parathion in sea and river water. Therefore, the third method which permits to carry out the infrared analysis, has been designed in order to establish more specific method for parathion determination. The Method 3. proposed for parathion determination is based on the solvent extraction, chromatography using heavy paper and infrared spectrophotometry. This method is also applicable to other insecticides illustrated in Table I, and the sample can be treated on a scale of 100 mg or more, if necessary.

Procedure: The steps of this method can be summarized as follows:

(1) Collection of parathion by liquid-liquid extraction. Parathion in the water sample

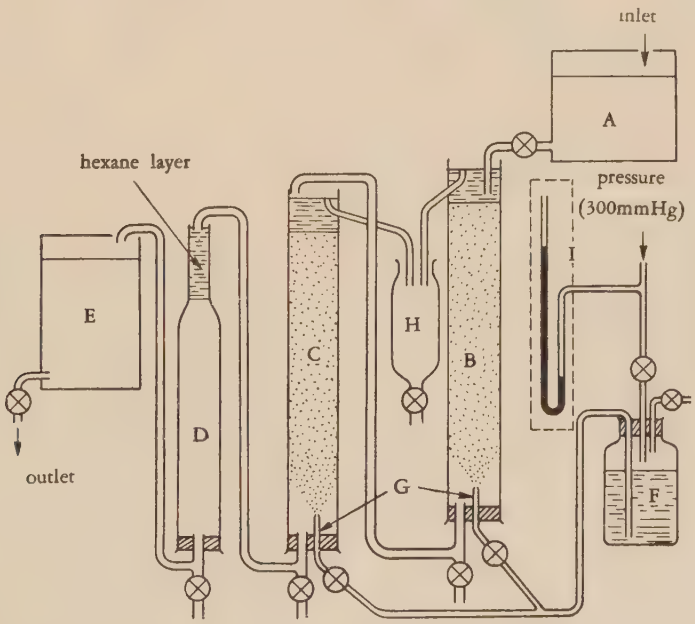


FIG. 1. Extractor for Liquid-Liquid System.

A, water sample tank; B & C, extraction column; D, separating column; E, separating tank; F, fresh solvent tank; G, nozzle; H, tank for extract layer; I, manometer.

can be quantitatively extracted into 2-4 l of the solvent (equal volume mixture of

1) Y. Sumiki, A. Matsuyama, H. Kato, Y. Chen and T. Yamauchi, *Bull. Agr. Chem. Soc. Japan*, **21**, 204 (1957).

TABLE I. R_F VALUES OF PARATHION AND RELATED COMPOUNDS

Compound (Mobile Solvent)	*n*-Hexane	Ethanol	Ethanol-Nitromethane (4 : 1 v/v)
(Stationary Solvent)	Ethylenechlorohydrine-Methanol (1:1 v/v)	Soybean oil	Soybean oil
Parathion	0.23	0.56	0.53
Methylparathion	0.10	0.65	0.63
4124 (Isochlorthion)	0.17	0.54	0.63
Ethyl 4124	0.36	0.45	0.41
Chlorthion	0.13	0.56	0.50
Ethylchlorthion	0.30	0.54	0.43
O-(2-Nitrophenyl)-*O*,*O*-diethylthiophosphate	(0.02), (0.06), 0.18, 0.32	0.60, 0.81	0.61, 0.76
EPN	0.22	0.40	0.37
Dipterex	0.02	0.79	0.81
Malathion	0.12	0.69	0.75
TEPP, technical	0, 0.03	0.71, 0.75	0.70, 0.75
Diaginon	0.03	0.63	0.60
Systox, technical	0.02, 0.10	(0.04), (0.64), 0.78	(0.04), (0.64), 0.79
Metasystox, technical	0.02, 0.04	0.74	(0.02), 0.74
Thimet, technical	(0.02), (0.11), (0.19), 0.75	0.56, (0.82)	0.46, (0.80)
Trithion, technical	(0.01), 0.64	(0.06), 0.28	(0.08), 0.38, (0.86)
DDVP, technical	0, 0.02, (0.05), 0.10	0.05, 0.82	0.05, 0.85
p,*p*'-DDT	0.74	0.26	0.28
γ-BHC	0.32	0.41	0.42
Orthophosphate	0	0	0
Pyrophosphate	0	0.01	0.02

Development of spot: by UV lamp irradiation, Hanes-Isherwood reagent and UV radiation[2], and indicator for chlorinated pesticides[3].
( ) indicates the light spot.

benzene and hexane) by passing 200 l of the water sample through the extractor at the rate of 100–120 l per hour. The solvent is introduced from a nozzle as a spray and allowed to pass gently through the extraction column in countercurrent wise against the water sample flow. This extractor can be operated in the working field or on boats where electric or any other motive power is unavailable.

(2) Concentration of extracted layer. The removal of the solvent is carried out at first by the vacuum distillation apparatus with a long neck distillating flask till the volume of the solution comes to about 200 ml and followed by carefull evaporation to oils using an agitating evaporator[1]. Ninety seven per cent of parathion can be recovered throughout the steps of extraction and concentration.

(3) Isolation by paper chromatography. Parathion in the extracts can be isolated from other insecticides by the paper chromatographic technique. Two separate chromatographic systems by descending method are employed in conjunction. The first uses soybean oil as a stationary solvent and either ethanol-nitromethane (4:1 v/v) or ethanol only as a mobile solvent. This system mainly serves to separate parathion and EPN from each other. The second uses ethylenechlorohydrine methanol mixture (1:1 v/v) as a stationary solvent and *n*-hexane as a mobile solvent. This system separates parathion from other insecticides except EPN. The spot of

2) J.A.A. Ketelaar and J.E. Hellingman, *Anal. Chem.* **23**, 646 (1951).
3) K.O. Keefe and P.R. Averell, *ibid.*, **23**, 1167 (1951).

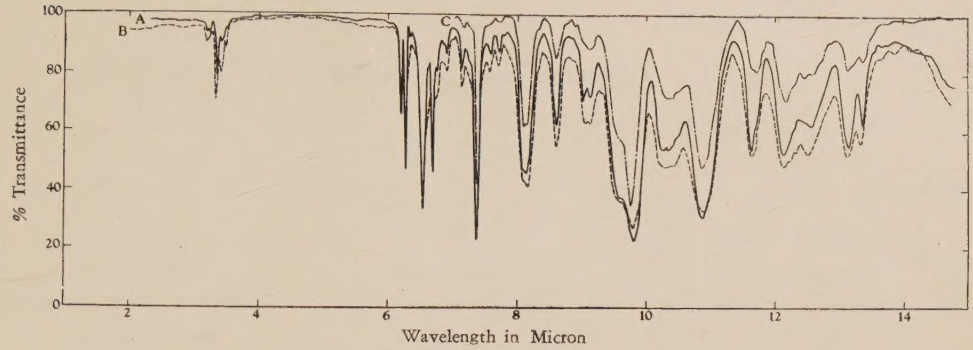


FIG. 2. Infrared Absorption Spectra of Parathion
A: Liquid film (unchromatographed) B: Liquid film (chromatographed) C: 2% in CS_2, 0.1-mm. cell

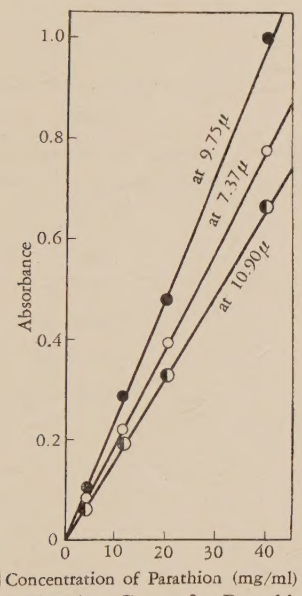


FIG. 3. Calibration Curve for Parathion in Carbon Disulfide Solution (in 0.1-mm cell).

parathion can be detected by ultraviolet lamp irradiation under the guiding of its authentic sample. In this study the thicker paper (Toyo Roshi No. 27 for oil filter press) is used in order that the sample may be treated on a scale of 100 mg or more. In the above chromatographic techique, further development of the paper chromatogram or liquid chromatography using a fraction collector can also be performed.

(4) Elution from paper chromatogram by ether

(5) Infrared spectrophotometry. A carbon disulfide solution can be used for qualitative identification in the 2- to 15-micron region except the 6.2- to 7.0-micron region (thickness at 0.1 mm.) and also for quantitative determination by measuring the absorption as 7.37, 9.75 and 10.90 microns. If only qualitative examination is required, the spectrum may be obtained without dilution.

(6) Quantitative measurement of amounts of parathion. It is preferable that parathion is determined by means of several methods such as gravimetry and colorimetry of *p*-nitrophenate ion[2,3], azo dye[1,4] and phosphorus[1] in paralled with infrared analysis.

The recovery in the analysis of the well-water sample by this presented method is 94%. The experiments of the analysis of the sea and river water samples are at present under way. The authors wish to thank the National Institute of Agricultural Science, the Agricultural Chemical Inspection Station of the Ministry of Agriculture and Forestry, Sumitomo Chem. Ind. Co., Toa Agr. Chem. Co., Sankyo Co. for supplying the samples.

Yusuke SUMIKI
Akira MATSUYAMA

Department of Agricultural Chemistry,
Faculty of Agriculture,
University of Tokyo.

Received August 5, 1957

4) P.R. Averell and M.V. Norris, *Anal. Chem.* **20**, 753 (1948)
5) R.S. Bandurski, *J. Biol. Chem.*, **193**, 405 (1951).
6) L.C. Mitchel, *J.A.O.A.C.*, **39**, 891 (1956).

Additions and Corrections

Vol. 21, No. 3.

page	column	line	for	read
160	synopsis	3	*Aqufoliaceae*	*Aquifoliaceae*
160	synopsis	5	β-sitosterol discussed	β-sitosterol was discussed
160	left	1	In previous	In the previous
160	left	8	show that	shown that
161	right	28	+16.81°	−16.81°
163	left	ref. 14)	Jungfeisch	Jungfleisch
163	right	ref. 25)	Yagishsta	Yagishita
164	right	2	α-amyrin (*α-Amyrin*	β-amyrin (*β-Amyrin*
164	right	15	shoud	should
164	right	23–24	"should be" *Viscum album* Linnaeus *var. coloratum* Ohwi.	should be "*Viscum album* Linnaeus *var. coloratum* Ohwi".
166	author's position		*Collega*	*College,*
166	left	4	Workers	workers
170	TABLE I	8,12,16,20	30 C	30°C
185	right	1	Formery	Formerly
185	right	2	amylase	amylase[9)]
191	right	−3	Laboratohy	Laboratory

Journal of the Agricultural Chemical Society of Japan

(Printed in Japanese)
Vol. 31, No. 7 (1957)

Journal of the Agricultural Chemical Society of Japan

(Printed by Japanese)
Vol. 31, No. 8 (1957)

PRINTED BY
KASAI PUBLISHING & PRINTING CO.
(Pan-Pacific Press)
MINATO-KU, TOKYO